

PHYSIOLOGICAL REVIEWS

VOLUME 22

1942

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BALTIMORE, MD.

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JOHNSON REPRINT CORPORATION

New York, New York

weight of the oxygen and $I.W.$ is a correspondingly smaller fraction of $I.L.$ On the other hand, if fat alone were being metabolized $I.W.$ would be slightly greater than $I.L.$ Mitchell and Hamilton (2) have shown that this ratio $\frac{I.W.}{I.L.}$ is also modified by the per cent of the total heat, removed by vaporization of water. To see clearly the effect of different respiratory quotients on this ratio one must deal with a fixed per cent of heat lost by vaporization of water. For example, when 25 per cent of the heat is lost by evaporation of water at an R. Q. of 0.707, $\frac{I.W.}{I.L.} = 101.6$ per cent; while at an R. Q. of 1.00, it is only 79.9 per cent. At the usual basal R. Q. of 0.825, $\frac{I.W.}{I.L.}$ is 91.2 per cent.

The effect of shifts in the amount of heat lost by vaporization may be seen from the following considerations. An individual in 24 hours absorbed 537 grams O_2 and excreted 573 grams of CO_2 and produced 1772 calories. The R. Q. was 0.777. His insensible loss of weight for the period was 757 grams. From Isenschmidt's equation, the $I.W.$ was 721 grams. He lost 23.6 per cent of the heat by vaporization of water $\left(\frac{721 \times 0.58}{1772}\right)$ and $\frac{I.W.}{I.L.} = \frac{721}{757} = 95.2$ per cent.

Assuming that the heat production and the metabolic mixture remain the same but the amount of heat lost by vaporization of water is now 47.2 per cent, $I.W.$ would be 1442 grams and therefore $I.L.$ would be 1478 grams and $\frac{I.W.}{I.L.}$ would be 97.6 per cent. Clearly the effect of change in per cent of heat removed by vaporization is much smaller than that caused by shifts in R. Q. As Mitchell and Hamilton have pointed out, at any given R. Q. as the per cent of heat removed by vaporization increases, $I.W.$ approaches $I.L.$ These relationships are summarized in their table 1 which is reproduced here.

PARTITION OF I. W. By definition $I.W.$ includes all of the water vaporized plus any water which leaves the skin in the liquid state. This bifold loss of water takes place when the heat production is very great and the environmental conditions do not favor evaporation. It may also occur in quiet states when the relative humidity approaches 100 per cent and the air temperature is high. Even under extreme conditions, this loss of liquid water is never more than a small part of $I.W.$ Accordingly under ordinary circumstances one has to deal only with vaporized water. Water is vaporized from both the respiratory tract and the skin.

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PHYSIOLOGICAL REVIEWS

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No. 1

THE INSENSIBLE LOSS OF WATER

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Water is being continuously evaporated from the lungs and skin. At times there is an additional loss from the skin in the form of liquid water. This insensible loss of water because of its magnitude, is an important fraction of the water excreted by the organism. Further, since it requires 0.58 calory to evaporate one gram of water, the vaporization of water is one of the means for removal of heat. This loss of water, both gaseous and liquid, is designated "the insensible loss of water." This phenomenon is the main cause of the insensible loss of weight.

It is more than 300 years since Sanctorius, suspended from one arm of a beam balance, observed that he lost weight progressively even though he eliminated neither urine nor feces. But the true nature of this "perspiratio insensibilis," as it was named by its discoverer, was not understood until comparatively recent times.

THE RELATIONSHIP OF INSENSIBLE LOSS OF WATER TO INSENSIBLE LOSS OF WEIGHT. Isenschmidt (1) expressed this relationship in the form of an equation:

$$I.L.^1 = I.W. + CO_2 - O_2.$$

It is evident from a consideration of Isenschmidt's equation that $I.W.$ can be calculated from $I.L.$ when the weights of CO_2 and O_2 are known. When the metabolic mixture is such that the weight of the carbon dioxide excreted equals the weight of the oxygen absorbed, $I.W. = I.L.$ This condition exists when the respiratory quotient is 0.725. As the proportion of carbohydrate in the metabolic mixture increases, the weight of the carbon dioxide becomes progressively greater than the

¹ $I.L.$ equals insensible loss of weight in grams. $I.W.$ equals weight of water vaporized in grams. CO_2 equals weight of carbon dioxide produced in grams. O_2 equals weight of oxygen absorbed in grams.

weight of the oxygen and $I.W.$ is a correspondingly smaller fraction of $I.L.$ On the other hand, if fat alone were being metabolized $I.W.$ would be slightly greater than $I.L.$ Mitchell and Hamilton (2) have shown that this ratio $\frac{I.W.}{I.L.}$ is also modified by the per cent of the total heat, removed by vaporization of water. To see clearly the effect of different respiratory quotients on this ratio one must deal with a fixed per cent of heat lost by vaporization of water. For example, when 25 per cent of the heat is lost by evaporation of water at an R. Q. of 0.707, $\frac{I.W.}{I.L.} = 101.6$ per cent; while at an R. Q. of 1.00, it is only 79.9 per cent. At the usual basal R. Q. of 0.825, $\frac{I.W.}{I.L.}$ is 91.2 per cent.

The effect of shifts in the amount of heat lost by vaporization may be seen from the following considerations. An individual in 24 hours absorbed 537 grams O_2 and excreted 573 grams of CO_2 and produced 1772 calories. The R. Q. was 0.777. His insensible loss of weight for the period was 757 grams. From Isenschmidt's equation, the $I.W.$ was 721 grams. He lost 23.6 per cent of the heat by vaporization of water $\left(\frac{721 \times 0.58}{1772}\right)$ and $\frac{I.W.}{I.L.} = \frac{721}{757} = 95.2$ per cent.

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Respiratory tract. The amount of water vapor leaving the respiratory tract is related to the temperature and water content of the inspired air and to the volume of air breathed. Benedict and Benedict (3), with their usual precision, determined separately the loss of water from the respiratory tract and skin. When the subjects breathed dry oxygen at room temperature the basal values for loss from the lungs varied from 8.8 grams to 12.05 grams per hour for one woman and two men. This was close to 40 per cent of *I.W.*

The relationship between lung ventilation and weight of water vapor lost from the lungs is seen when subject B increased his ventilation from 369 liters to 891 liters per hour. The corresponding lung waters were 12.5 grams and 23.58 grams.

TABLE 1 (2)

The percentage of the insensible weight loss represented by vaporized water for various values of the respiratory quotient

| PERCENTAGE OF HEAT LOST AS VAPORIZED WATER | PERCENTAGE LOSS AT RESPIRATORY QUOTIENT OF | | | | | | |
|---|--|------|------|------|------|------|------|
| | 0.707 | 0.75 | 0.80 | 0.85 | 0.90 | 0.95 | 1.00 |
| 20 | 102.0 | 06.8 | 91.6 | 87.0 | 82.9 | 79.3 | 76.1 |
| 25 | 101.6 | 97.4 | 93.1 | 89.3 | 85.8 | 82.7 | 70.0 |
| 30 | 101.3 | 97.9 | 94.2 | 90.9 | 87.9 | 85.2 | 82.7 |
| 35 | 101.1 | 98.2 | 95.0 | 92.1 | 89.5 | 87.0 | 84.8 |
| 40 | 101.0 | 98.4 | 95.6 | 93.0 | 90.7 | 88.4 | 86.4 |
| 45 | 100.9 | 98.6 | 96.1 | 93.8 | 91.6 | 89.6 | 87.7 |
| 50 | 100.8 | 98.7 | 96.4 | 94.3 | 92.4 | 90.5 | 88.8 |

In 1930, Jores (4) obtained essentially similar results, using the same technique, for the partition of water vapor between lung and skin.

Since in both cases the subjects inspired dry oxygen it is instructive to examine the values obtained when room air is breathed. Galeotti (5) found that at a room temperature of 12.5°C. the water content of the expired air was 0.0326 gram per liter when the water content of the inspired air was 0.0034 gram per liter (relative humidity 30 per cent). The difference is approximately the amount of water contributed to the expired air by the respiratory tract, namely, 0.0292 gram per liter. When the temperature was the same but the water content of the inspired air was 0.0075 gram per liter (relative humidity 68 per cent), the expired air contained 0.0322 and the difference was 0.0247 gram per liter. The effect of relative humidity is evident. The temperature effect is seen from average values when the air temperature was 24°C.

and the water content of inspired air was 0.0115 gram per liter (relative humidity 50 per cent), the average water content of expired air was 0.0366. The difference is 0.0251. Unfortunately the author did not study the effect of the lower temperature at this relative humidity. However, if one averages the values obtained at 12.5°C. for the two humidities used (30 and 68 per cent) one obtains a value of 0.0270 gram per liter. If this number is compared with 0.0251, it is seen that increasing the temperature of the inspired air with relative humidity unchanged, causes a lessened loss of water from the respiratory tract.

Further information about the effect of the moisture of the inspired air is reported by Adachi and Ito (6). Their data are especially interesting because they show that the vaporization from the skin increases to compensate for a diminished loss from the lungs. When the subject breathed dry air, the loss per hour from the lungs was 7.90 grams and from the skin 21.56. In contrast, the subject responded to inspired air saturated with moisture by losing only 4.30 grams per hour from the lungs, but the evaporation from the skin increased to 23.6 grams.

While water content of the inspired air has more effect upon the loss of water from the respiratory tract than temperature, both effects are small when the subject is quiet.

There has been confusion in the literature concerning the question whether the expired air is saturated with water vapor. This apparently arose because it was assumed that the temperature of expired air is 37°C. Galeotti (7) measured the temperature of expired air by means of a thermocouple at room temperature from 16 to 35°C. and found it to vary between 34.4 and 35.7°C. At these temperatures it was 90 per cent saturated. The water content was only 78 per cent of that of saturated air at 37°C.

This loss from the respiratory tract is then controlled by the physical conditions of the environment, and by only one biological factor—the lung ventilation. The volume of air breathed, barring voluntary effects, is related directly to heat production.

Skin. The evaporation from the skin is affected by the following factors: environment, humidity, temperature and air currents, circulation of blood through skin, character of skin covering, water content of body.

Investigators have recorded these effects upon either *I.L.* or *I.W.*, not upon the loss from the skin separately.

Humidity. For the nude male subject in the basal state when the environmental temperature was fixed at 28°C., Wiley and Newburgh

(8) obtained the following values for *I.W.* per hour: 22, 17, 15, 14 grams for relative humidities of 20, 40, 60 and 80 per cent respectively. On the other hand, the subject, comfortably clothed, vaporized the same amount of water when the relative humidity varied from 20 to 60 per cent. This was confirmed by Van Harreveld, Grutterink and Noyons (9) who found little effect on *I.L.* for variations of plus or minus 35 per cent relative humidity; and by Ginandes and Topper (10) who recorded not more than 10 per cent variation in *I.L.* of children when the relative humidity varied from 19 to 74 per cent. However, the *I.L.* was lessened by humidities above 74 per cent and increased when it was less than 19 per cent. Clearly, then, the *I.W.* of the comfortably clothed subject is affected only by extreme dryness or wetness of the air.

Temperature. Figure 1 (8) shows the interrelationship between heat production, skin temperature and total *I.W.* at room temperatures from 18°C. to 40°C. at a relative humidity of 20 per cent. The male subject was nude, fasting and recumbent. It will be seen that even though the heat production was greatest at 18°C., at this temperature the *I.W.* was at its lowest value. At this point the skin temperature was at its lowest level also. As the skin temperature decreased from 34°C. to 29°C., the *I.W.* also decreased slowly and uniformly. The total decrease was about 115 grams.

This ability of the organism to decrease vaporization of water through the skin is even greater than it appears since with the same relative humidity the amount of water evaporated from the lungs must have increased as the environmental temperature fell.

Further inspection of figure 1 shows that the *I.W.* began to increase rapidly at about 30°C. This abrupt change implies that a second factor has now been added to the mechanism, which permitted an increase in the vaporization of water that amounted to 465 grams per 24 hours per degree change in environmental temperature at the higher temperatures, as contrasted with 11 grams at the lower. This huge increase is attributable to the activity of the sweat glands.

The relative contribution to the maintenance of the homeothermic state by the integument, aided and unaided by the sweat glands, may be realized from studies on individuals without sweat glands.

In 1911 Loewy and Weehselman (11) showed for the first time that the human being can lose water through the skin in the absence of sweat glands. Examination of 600 sections of axillary skin did not reveal a single sweat gland. They studied the response of the skin of the leg by enclosing it in a metal cylinder so arranged that the water vaporized from the skin

could be measured. They found that the patient lost about the same amount of water vapor per square meter and hour as the control in the basal state, and they concluded that simple physical diffusion of water vapor through the normal skin occurs and that this is the total loss from the skin when no special demands for removal of heat exist. The sweat

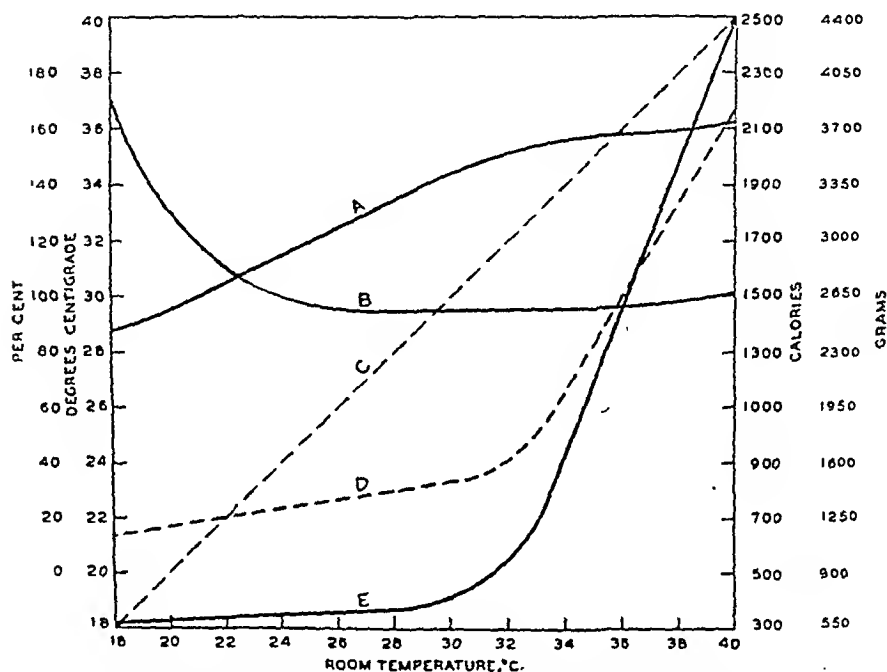


Fig. 1. Nude subject. The effect of room temperature on skin temperature, basal heat production and the dissipation of heat by the vaporization of water at a relative humidity of 20 per cent.

A. Skin temperature in degrees centigrade. B. Basal heat production in calories per 24 hours. C. Rate of increase in room temperature. D. Heat lost by the vaporization of water in per cent of the total heat dissipated, or grams of water vapor per 24 hours. E. Heat dissipated by the vaporization of water in calories per 24 hours.

glands are only intermittently active, i.e., during great muscular activity and in hot environments.

Richardson (12) studied a 14 year old boy, examination of whose skin revealed no sweat glands. At rest in the calorimeter when the temperature was 24 to 25°C. the amount of water vaporized compared favorably with the control. The extra heat produced by exercise at this same

calorimeter temperature was eliminated by the patient nearly as well as by the control but the per cent removed by vaporization was somewhat less on the part of the patient. But when the patient, except for his head, was enclosed in a bag and the temperature of the air passing through it was 48 to 49°C., he lost 33 grams per hour insensibly. The control under the same conditions lost 336 grams. By separating the loss from the skin and lungs it was shown that the patient evaporated 22 grams per hour from the skin at rest when exposed to an environmental temperature of 24.5°C. At the same time, 9 grams were lost from the respiratory tract.

The most recent study of this condition was made by Sunderman (13). His two patients and two controls were exposed to environmental temperatures of 43.3°C. for 30 minutes. The oral temperatures of the controls remained normal and the *I.Ls.* were 262 and 360 grams. But the oral temperatures of the patients rose to 101.4 and 102.0°F., and they lost only 22 and 15 grams insensibly. The basal insensible losses of these two patients were normal.

Air currents. Benedict and Benedict (3) recorded the loss of water vapor from the skin on the part of a nude female subject in the resting state at an environmental temperature of 21°C., before and while exposed to air movements produced by a blower. The values were 11 grams and 10.2 grams per hour.

DuBois (14) in carefully conducted experiments recorded the separate fractions of heat loss by means of the calorimeter. The nude male subject was in the post-absorptive state, lying quietly and exposed to an environmental temperature of 29°C. Without the fan he lost 30 grams *I.W.* per hour which accounted for a loss of 30 per cent of the heat. With the fan, the *I.W.* was 37 grams—a loss of 26 per cent of the heat because the total loss of heat had increased. This is especially instructive since in quiet air only 11 per cent of the heat was removed by convection, whereas the fan increased this loss to 33 per cent.

Sex differences have been observed by Hardy (15) and co-workers. Nude female subjects, when exposed to the same conditions as nude men in the calorimeter, showed a slightly lower *I.W.* per square meter per hour at temperatures from 22 to 29°C. At the latter temperature sweating began in the women, but was delayed until 31°C. for the men. The larger question of heat regulation as related to sex is carefully studied by these authors.

Clothing. Having now dealt with the responses of the nude subject to environmental temperatures, the protective effect of clothing will be

shown. It is obvious that clothing will not prevent the onset of sweating at the point at which it would occur in the nude subject. However, Wiley and Newburgh (8) showed that protection by means of heavy pajamas caused their subject to have a constant *I.W.* for environmental temperatures from 24 to 30°C. Below 24°C. he behaved as did the nude subject in regard to *I.W.* Had he been further protected against cold it seems evident that the range over which *I.W.* would have been constant would have reached much lower temperatures.

Circulation through skin. The organism may be thought of in terms of a moist protoplasmic mass enclosed in an integument that permits only a limited amount of water to diffuse outward. The actual amount of diffusion is related to the volume of the circulation through the skin and to its temperature; but the organism is capable of increasing *I.W.* far beyond this limited amount through the activity of the sweat glands.

Wiley and Newburgh's (8) subject was able to vaporize about 18 grams per square meter an hour before sweating took place. But the organism frequently loses by diffusion less than the greatest possible amount of water. For example, this same subject, whose heat production had been increased about 33 per cent above the basal level, vaporized 17.7 grams per square meter per hour when the skin temperature was 32°C. At the same skin temperature in the basal state he vaporized only 16 grams. This restriction is brought about by changes in the volume flow through the skin. One effect of lessening the flow is fall in temperature of the surface of the body. When this took place in Wiley and Newburgh's (8) subject the decrease in water vapor at the lower skin temperature followed closely the predicted values corresponding to the change in vapor pressure. Barbour (16) reached the same conclusion, working with cats. He also showed that the hemoconcentration which occurs as part of the response to cold did not affect vapor pressure significantly. But since different actual amounts of water may be vaporized per unit of surface at the same skin temperature, the latter cannot be the only controlling factor. It is believed that the integument, flooded with blood, can lose water vapor more easily than when it is caused to become relatively dry by curtailment of the circulation. The volume flow, in its turn, is controlled by the vasomotor apparatus. Hardy and Oppel (17) have shown how extremely sensitive the skin is to changes in environmental temperature; and these responses are signals to the vasomotor center.

Return to the sweating apparatus affords the opportunity of appreciating to what extent the integument resists the loss of body water. The subject studied by Wiley and Newburgh (8) when exposed to a room

temperature of 40°C., vaporized 107 grams of water per square meter and hour. The skin temperature was 36°C.; however, when he was exposed to an environmental temperature of 29°C., he vaporized only 18 grams. The skin temperature was now 34°C. These differences in skin temperatures account for only a very small part of the change. The sweat glands, in the first case, pumped water onto the skin and the environment quickly carried it away. As organisms gradually passed from the waters to the dry land, it was necessary to develop means of conserving water. This was accomplished in part by decreasing the permeability of the skin. However, with the development of the homeothermic state, a mechanism working in the opposite direction came into existence to protect the organism from overheating when the metabolism was accelerated.

The dog, almost devoid of sweat glands, pants to rid himself of heat when man would sweat. Polypnea is effective for two reasons. First the rapid fanning of the wet tongue is an efficient method of vaporizing water, but the area is so small that it may not be sufficient. A second gain is made by virtue of the shallowness of the respiration. Christie and Loomis (18) have shown that rapid shallow breathing is a successful method for the elimination of heat without causing a serious loss of CO₂.

WATER CONTENT OF BODY. Manchester, Husted and McQuarrie (19), in 1931, believed that they had shown that diminution in water content of body reduced *I.W.*, but Newburgh and Johnston (20) were not convinced that the data were gathered with sufficient care to establish the point. They found that reduction of the body water by approximately 6 per cent did not reduce *I.W.* Their subject, who weighed 60 kilos, was caused to lose 2863 grams by withholding water and feeding 10 grams of NaCl daily. This represents a loss of approximately 6 per cent of his body water. Dehydration of this magnitude did not significantly affect *I.W.* Levine and Wyatt (21) determined the basal *I.L.* of the same infants before and after dehydration. The latter state was accompanied by reduction of 11 per cent in *I.L.* but, as they point out, the large fall in *R. Q.* that is observed in dehydrated infants may account for 8 per cent in the shift in *I.L.*

Hall and McClure (22) confirmed Newburgh and Johnston in their conclusion that *I.W.* is not affected by dehydration of 6 per cent or less, but showed that greater dehydration did lower *I.L.* Even though there has been a great deal of controversy about this question in the past, the experiments of Hall and McClure were conducted with such care that their conclusions may be considered final.

They next took up the question of an effect on *I.L.* of flooding the

body with water. They found that ingestion of as much as 3700 cc. of water in 3 hours did not change the rate of *I.L.* Ginandes and Topper (10) likewise observed no effect in children from ingestion of large amounts of water. However, when they (23) gave 5 to 15 grams of NaCl in capsules to 9 children, 6 to 10 years of age, they invariably observed a marked reduction in *I.L.* from 15 to 30 per cent. The heat production remained unchanged. This reduction began about one and one-half hours after administration of the salt and lasted about two hours. Gilman and Barbour (24) earlier demonstrated a similar response in rabbits and recorded simultaneous wide shifts in osmotic pressure of the blood. However, Barbour (16) pointed out in a later publication that such increases in osmotic pressure could not account quantitatively for the effect on *I.L.*

It should be noted that Gilman and Barbour (24) gave 1.7 grams of NaCl per kilo, and that Ginandes and Topper (23) gave from 0.3 to 0.7 gram per kilo. However, when Hall and McClure (22) gave about 0.2 gram NaCl (1200 cc. 1 per cent sol.) to adult human subjects, they were unable to observe any effect on *I.L.*

How does the extra accumulation of fluid in the body which occurs in edematous patients affect *I.W.*?

Beginning with a publication in 1929, Zak (25) has maintained that patients suffering from congestive heart failure are able to absorb water from the air. In response to adverse criticism, he later stated (26) that this phenomenon could not be demonstrated in all edematous cardiacs and that weighings had to be made every half hour because an insensible gain in weight might take place only once in many hours. In 1939, his pupil, Neurath, (27) published observations which appeared to him to further substantiate the possibility of this "negative insensible perspiration." We have difficulty in accepting his evidence based on two cases. The second one was an obese but otherwise normal individual who, following a morning weighing, received a large dose of mercurial diuretic intravenously. During the next ten hours, according to the record, no urine was voided. He lost 2790 grams insensibly. During the next 13 hours, the urine amounted to 1568 grams and he gained 788 grams insensibly. We are puzzled by the anuria since the previous subject produced 1059 grams of urine and again 1087 grams in the 5 hours immediately following the diuretic.

On the other hand, Heller (28), Jores (29), Gereb and Laszo (30), Magendantz and Stratmann (31), Gabriel and Kahler (32) and later, Kahler and Schmidt (33), and Kesterman and Schleining (34) never

recorded an insensible gain in weight in the many edematous patients studied by them.

A study made in this laboratory by R. H. Freyberg (35) of a far advanced nephritic disclosed entirely normal *I.L.* throughout. The pertinent data are brought together in table 2. The patient had been freed of edema a few days earlier. The diet was identical throughout. The daily fluid intake varied only between 3800 and 4000 grams. It will be seen that the small variations in *I.W.* are of the same order of magnitude when he was edema-free or whether he was gaining or losing

TABLE 2

| DATE | BODY WEIGHT | <i>I.L.</i> | <i>I.W.*</i> | |
|---------|--------------|--------------|--------------|---------------------------|
| | <i>kilos</i> | <i>grams</i> | <i>grams</i> | |
| Nov. 12 | 53.916 | 1,209 | 1,138 | |
| 13 | 53.850 | 1,103 | 1,031 | |
| 14 | 53.818 | 1,182 | 1,116 | |
| 15 | 53.998 | 1,227 | 1,157 | 10 gms. NaCl |
| 16 | 54.953 | 1,184 | 1,113 | 10 gms. NaCl |
| 17 | 56.009 | 1,060 | 987 | 10 gms. NaCl |
| 18 | 57.073 | 1,200 | 1,114 | |
| 23 | 55.555 | 1,114 | 1,041 | 8 gms. NH ₄ Cl |
| 24 | 52.220 | 1,172 | 1,101 | 8 gms. NH ₄ Cl |
| 25 | 54.722 | 1,111 | 1,039 | 8 gms. NH ₄ Cl |
| 26 | 54.487 | 1,219 | 1,148 | 8 gms. NH ₄ Cl |
| 27 | 53.584 | 1,231 | 1,161 | 8 gms. NH ₄ Cl |

* ($\text{CO}_2 - \text{O}_2$) calculated from metabolic mixture.

edema. We know of no unequivocal evidence that *I.W.* is affected by gain or loss of edema fluid.

Is there any reasonable explanation for the alleged insensible gain in weight? Two suggestions have been made. The first one is concerned with absorption of water by the skin itself. A search through the literature has disclosed no satisfactory experiments which show how much water can be taken up from a water bath or moist air. The former has been studied by Moog (36) and Eimer (37) but the data are not satisfactory. It is clear, however, that the organism after being depleted of water continues to lose weight insensibly when immersed in water. Moreover, Wiley and Newburgh's (8) nude subject, exposed to an atmosphere whose relative humidity was 20 per cent and whose

temperature was 28°C.; had an *I.W.* of 21.6 grams per hour. At 80 per cent humidity, at the same temperature, he lost 14.4 grams per hour. They attributed this decrease to the increased vapor pressure of the atmosphere. From the meagre evidence at hand it seems highly improbable that the human skin can absorb significant amounts of water from moist air. We find no satisfactory evidence to support the view that any water can be secreted inward.

The second suggestion assumes that oxygen is retained in the production of glucose from non-carbohydrate precursors (Silva-Mello, 38). Obviously the new glucose would have to be retained. Heller (39) calculates that this cannot account for an insensible gain in weight.

There are many pitfalls in the measurement of *I.L.* Mattresses, bedding and clothing absorb and lose water rapidly when exposed to changing humidities. The common use of wooden bed frames is objectionable since they also absorb and lose water. The thirst caused by restricted intake of fluid tempts patients to obtain water surreptitiously. All of these things should be borne in mind when evaluating the data in this field. It does not as yet seem necessary to accept the proposition that either the normal or abnormal individual ever fails to exhibit an insensible loss of weight.

RELATION TO HEAT ELIMINATION. The use of the respiration calorimeter necessitated the collection of the water vaporized as a means of measuring the heat removed by this process. As early as 1907, Benedict (40) reported that an average value of 22 per cent of the total heat eliminated, was removed by vaporization of water with a variation from 19 to 30 per cent. The per cent was essentially the same for the basal state and after food. Later Soderstrom and Du Bois (41) published a large number of observations which ranged from 21 to 32 per cent with an average of 25 per cent. Levine and Wilson (42, 43) obtained similar values for infants and children.

These observations make it clear that the comfortable quiet individual rids himself of an approximately fixed percentage of the heat of his metabolism by vaporization of water.

Since in the basal state *I.W.* is almost a constant portion of *I.L.*, it is evident that the latter may be employed as an indirect measure of total heat loss. Benedict and Root (44) presented an excellent review of the earlier literature and they formulated a table for predicting basal heat production from basal *I.L.* obtained by consecutive 10 to 15 minute periods. In 1930, Levine and Marples (45) published a satisfying treatment of this general question. They analyzed statistically 196

cases in which there were available simultaneous measurements of heat production and basal *I.L.* The data for adults were obtained in the Russell-Sage calorimeter (41) and those for infants and children in the respiration chamber at the New York Nursery and Child's Hospital (46, 47).

The high coefficient of correlation, 0.9417 ± 0.0064 , shows the close relationship between the *I.L.* and heat production. The smoothed curve derived from plotting these two variables against each other takes the form of a straight line which may be used for predicting heat from *I.L.* Several other investigators (4, 48, 49, 50) have studied this same relationship. A study of Levine and Marples' data reveals that essentially the same per cent of heat is removed by vaporization of water at all levels of heat production. These values as calculated by Heller (49) are 27.6 per cent at 500 calories and 24.2 per cent at 2,000 calories. Ginandes and Topper (50) in 56 observations on 27 children from 4 to 15 years of age, obtained a line whose slope resembled that of Levine and Marples but at the lower end, 32.6 per cent of the heat is removed by vaporization, whereas at the upper end the per cent is 25.7. Heller and Schwartz's (51) curve is identical with that of Levine and Marples at the upper end of the line in respect to per cent of heat vaporized but the slope of their line is a little steeper. It should be noted that their material included increases in heat production produced by food and exercise.

On the other hand, if one plots in the same way the data of the following investigators separately, Benedict and Root (44), Jores (4), Heller (49) and Laszlo and Schurmeyer (48), one obtains a series of divergent lines which depart widely from Levine and Marples. This means that the per cent of total heat lost by vaporization of water must have varied widely. Thus, at heat productions of 900 calories in Benedict and Root's series, the per cent is 19.4, whereas at 2,000 calories it is 31.3. If the line is continued downward, the *I.L.* would disappear just below 500 calories as pointed out by Heller (49). It should be noted that this latter group of investigators used for the most part abnormal subjects.

Newburgh and his associates (52) compared the 24-hourly *I.W.* with heat production of normal subjects determined by indirect calorimetry. In 29 experiments on 9 subjects they obtained an average value for the percent of heat lost by vaporization of water of 25.1 with a range from 21.2 to 27.7 per cent. When the *I.L.* of these subjects is plotted against the heat productions, the slope of the line thus obtained corresponds

almost exactly with that of Levine and Marples. Therefore, it seems to be true that not only does the human organism tend strongly to rid itself of close to 25 per cent of its heat by vaporization of water in the quiet, comfortable state from infancy through old age, but that the normal adult follows the same principle throughout the 24 hours. Levine and Wheatley (53), in a careful study, were unable to demonstrate this relationship for the 24 hours in infants. Since this relationship presumes that the subject is comfortable as regards the environment it is evident that one cannot know whether this condition exists in the case of infants.

Since it is in general true that 25 per cent of the total heat is lost by vaporization of water and since it is also true that heat production may be predicted from *I.L.* (obtained under carefully controlled conditions), it follows that the usual shifts in *R. Q.* do not change the relationship between *I.W.* and *I.L.* significantly. At present, the best data for prediction of heat from *I.L.* is represented by Levine and Marples' (45) smoothed curve.

I.W. in lower species. It has been learned from the study of man that the percent of heat lost by vaporization of water is closely related to the environmental temperature and that in the "comfort zone" approximately 25 per cent of the heat is so lost. Since it is manifestly impossible to know when an animal is comfortable, the investigator is at a serious disadvantage. The literature records the percent of heat lost by vaporization for various temperatures but does not permit one to conclude what the percent is at the ideal temperature, for most species. However, it is well known that there is a narrow range of environmental temperatures over which the basal heat production has a constant minimal value (thermic neutrality). It may well be true that measurements of *I.W.* well within this range would reveal the same constancy of percent of heat lost by vaporization of water as occurs in man. It was shown early by Rubner (67) that the dog responded like unclothed man to environmental temperature. When exposed to 25°C. his dogs lost 24.4 per cent of the heat by vaporization of water, whereas at an environmental temperature of 15°C., the percent was 13.8. It should be noted that both Greene and Luce (57) and Lee (60) did study their animals close to thermic neutrality and their data are therefore of special value.

In many of the species the number of observations is too few and experimental conditions were insufficiently controlled.

Table 3 brings together the data in the literature.

RELATION TO WATER EXCHANGE. In order to compare quantitatively the water available to the organism with the elimination of water, one must, in addition to measuring that which is ingested, calculate the amount produced by oxidation and also the preformed water released by catabolic processes or fixed by anabolic ones. On the outgoing side the value of *I.W.* must be determined, in addition to measuring the water content of the urine and stool. The water of oxidation is derived, by means of factors, from the metabolic mixture. The composition of the latter may be calculated from total heat production derived from *I.L.* and the urinary N by methods discussed by Newburgh and his

TABLE 3

| SPECIES | INVESTIGATOR | ENVIRON- MENTAL TEMPER- ATURE | PER CENT OF HEAT LOST BY VAPORIZA- TION OF WATER | |
|---------------------|-------------------------|--|--|---------|
| | | | Range | Average |
| | | °C. | | |
| Rat, fasting..... | Greene & Luco (57) | 25-31 | 20.6-26.8 | 23.8 |
| Rat, 24 hours..... | Greene (58) | 23-27 | 20.8-21.6 | 21.3 |
| Mouse..... | Benedict & Lee (59) | 28 | | 18 |
| Rabbit..... | Lee (60) | 28-29 | 17.7-29.7 | 24.6 |
| Goose..... | Benedict & Lee (61) | 16-29 | 15-40 | |
| | | 18-22 | | 16 |
| | | 16.9-23.2 | 17-34 | 26.1 |
| Cow..... | Ritzman & Benedict (62) | 8.5- 9.8 | 14-15 | |
| | | Uniform | 22.3-37.3 | 27.6 |
| Cow..... | Kriss (63) | 24.5 | 26.7-34 | 30.8 |
| Steer, sheared..... | Mitchell (2) | 20-24.7 | 17.2-19.1 | 18.3 |
| Elephant..... | Benedict (64) | 25 | | 24.4 |
| Dog..... | Rubner (65) | | | |

associates (52). The preformed water is then calculated by comparing the diet with the metabolic mixture.

The nature of *I.W.* has already been discussed. It has been pointed out that it may be determined directly in the calorimeter or that it may be derived from *I.L.* From the strictly quantitative aspect the most satisfactory method is the determination of CO₂ output and O₂ absorption by means of the respiration chamber. Then *I.W.* may be derived from *I.L.* by employing Isenschmidt's equation. Since such facilities are limited and restricted in length of period and in activity of the subject, they cannot be widely used. Accordingly, Lavietes (54) and Newburgh and associates (52, 55, 56) discussed the manner in which

CO₂ and O₂ can be calculated from the metabolic mixture. Since these calculations assume carbohydrate balance and that the percent of the heat lost by vaporization of water is fixed at 25 per cent, errors of unknown magnitude may be present.

On the other hand, can it be assumed that *I.W.* is a fixed percent of *I.L.* under certain conditions? Consideration of Levine and Marples' (45) prediction where approximately 25 per cent of the heat was lost by vaporization of water, reveals that close to 94 per cent of *I.L.* is *I.W.* Furthermore, a study of data obtained in this laboratory (68) on a normal man fed a normal maintenance diet (P. 91, F. 186, CHO. 241 grams) for 18 days showed that $\frac{I.W.}{I.L.} \times 100 = 93$ per cent. When he was fed a supermaintenance diet (P. 89, F. 413, CHO. 445 grams) for 15 days the $\frac{I.W.}{I.L.} \times 100 = 88$ per cent. Since the first diet approximates the usual mixed one, the relation between *I.L.* and *I.W.* would be expected to be the same as the average one for the group reported by Levine and Marples. Accordingly, when ordinary maintenance diets are fed, it may properly be assumed that *I.W.* is 93 or 94 per cent of *I.L.* Supermaintenance high carbohydrate plans lower the percent toward 88. It is unlikely that it will ever be significantly lower. If, however, the carbohydrate metabolized is low because of dietary restriction, $\frac{I.W.}{I.L.}$ approaches one.

SUMMARY

The water vaporized from lungs and skin removes heat and also plays an important part in water exchange.

Its relationship to total insensible loss of weight and its partition between skin and lungs is discussed.

The effect of environmental temperature and humidity, air currents, clothing, blood flow through the skin, is dealt with.

The part played by the sweat glands is analyzed.

The relationship of water content of body to insensible water is discussed.

The use of the insensible water as a measurement of heat production is analyzed, as well as its place in the quantitative measure of water exchange.

The percent of heat removed by vaporization of water in lower species is noted.

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HEMOGLOBINURIA

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Hemoglobinuria, dramatically associated with a variety of pathological conditions, has doubtless always been a source of wonder and amazement to patients as well as a valuable diagnostic guide to clinicians. Experimentally it was first described about the middle of the nineteenth century and problems relating to the phenomenon have intrigued investigators ever since.

In all the earlier studies and in many that have appeared within relatively recent years, the emphasis is placed on the site rather than on the actual mechanism of renal hemoglobin elimination. The literature contains a group of conflicting theories, each of which tends to be documented in a self-perpetuating fashion and few attempts have been made to explain the phenomenon of hemoglobinuria in the light of facts uncovered in other fields of renal research. Due to the great variety of animals and methods used and to conclusions drawn from purely qualitative and often incomplete studies, comparison of many of the data in the literature is often difficult or impossible.

Experimental physiologists have presented and consistently substantiated the concept that, "beyond reasonable doubt, the central process in the formation of urine by the glomerular kidney is the separation at the glomerulus of a *protein free filtrate* of plasma, containing the diffusible constituents in the same concentration as in the plasma water, i.e., glomerular filtration" (42, Shannon, p. 63). This modern concept of renal function implies that the glomerular membrane, acting as a sieve, restricts the transfer of all molecules which are greater in diameter than the pores in the membrane. The filtration theory is supported by all the findings on the excretion of substances having a molecular weight of 15,000 or less (apparent molecular weight of inulin (8)) but does not attempt to explain the excretion of a large molecule like hemoglobin, except as a result of pathological glomerular changes. No recent attempt has been made to present an explanation of the fact that large molecules are excreted by the kidney, in apparently normal mammals,

which is adequately fitted into the theory which maintains as one of its major premises that these molecules *cannot* escape by way of the glomerulus.

Since in cases of experimental and clinical hemoglobinuria there is no reason to suspect any pre-existing kidney pathology, the explanation of the phenomenon must lie either in a transient injury caused by the hemoglobin or in some mechanism which is in harmony with the modern concepts of renal function.

The present review, limited to a critical survey of the experimental findings relating to hemoglobinuria, is an attempt to reach a clearer understanding of the factors underlying the elimination of hemoglobin by the kidney.

Numerous histological studies have demonstrated the presence of hemoglobin or its iron-containing breakdown products as granules of varying size in the epithelium of the convoluted tubules, following the intravenous injection of hemoglobin (6, 17, 24, 28). Minute brown granules are observed immediately after the initiation of hemoglobinuria, at which time they do not give a Prussian blue reaction for iron but may be stained by various methods which are specific for hemoglobin (15, 24, 28). After a lapse of several days or following repeated injections of hemoglobin the granules in the epithelial cells of the convoluted tubules appear larger and readily stain for iron (6). Such findings indicate that this portion of the kidney plays some part in the process of hemoglobin elimination and for years a controversy has existed as to whether the tubular activity is one of excretion or reabsorption.

The early exponents of the tubular excretion theory argued on a purely theoretical and inadequate experimental basis that this was the only mechanism involved (31). More recently the question of a dual process of glomerular filtration plus tubular excretion has been advocated by some observers (15, 17). In reviewing this work no definitely positive evidence of tubular excretion can be found and it would seem, in most instances, that an interpretation of the findings, in favor of reabsorption, could equally well be made. Also, evidence derived from the study of lower vertebrate forms indicates that such conclusions probably are not valid. It has been shown that directional movements of granules in epithelial cells cannot be established on histological bases (20, 27), that the aglomerular nephron does not eliminate hemoglobin even after injury with mercuric chloride (5), and that the basilar portions of the renal tubular cells of the toad are impermeable to large negatively charged colloidal molecules (16, 28).

Evidence favoring the belief that the hemoglobin, found in the tubular epithelial cells, has passed through the glomerular membrane and subsequently been picked up from the tubular lumina is more decisive. The more cautious studies of early morphologists led them to conclude that hemoglobin was never found in the tubular epithelium unless it could also be demonstrated in the glomerular capsule (23). Much of the early confusion was doubtless due to the fact that hemoglobin is readily observed in the capsular spaces at the height of hemoglobinuria but is not demonstrable at later stages due to the increased dilution. Further indirect proof of glomerular elimination is given by the fact that hemoglobinuria occurs in dogs in the presence of severe tubular damage caused by mercury poisoning (19), and also by the fact that in the course of a study of induced hemoglobinuria in humans, the rate of excretion of hemoglobin was found to be markedly elevated in cases of known glomerular damage (18). Finally an implication of simple glomerular filtration without consideration of tubular activity has been frequently assumed by workers whose research touches only secondarily upon the mode of hemoglobin excretion (9, 21, 45).

Although it is generally conceded that hemoglobin escapes through the glomerulus there is little agreement regarding the way in which it does so. Since serum albumin with a molecular weight (67,000) approximately the same as hemoglobin (68,800) does not normally appear in the urine, certain investigators have proposed that hemoglobin escapes through the glomerulus only as the result of an induced transient injury (9, 14). It cannot be denied that some increase in glomerular permeability is frequently associated with hemoglobinuria. Variable degrees of albuminuria occurring simultaneously with the excretion of the foreign protein egg white (1, 22) and hemoglobin (18, 35, 40), which in the free state is not a normal constituent of plasma, have been reported. The simultaneous excretion of ferri-ammonium citrate with carboxyhemoglobin (17) and egg albumin (2) has also been adduced as evidence of glomerular injury. The results of these studies are not consistent and since ferri-ammonium citrate may be found normally in the glomerular fluid (20), together with the presence of toxic impurities in many samples of this salt (43), the importance of such a line of investigation tends to be diminished.

A careful evaluation of all the evidence at hand indicates that glomerular injury is not the fundamental mechanism of hemoglobin excretion. Numerous studies have shown that the intravenous injection of carefully prepared, stroma-free hemoglobin solutions had no systemic

pharmacological activity (26, 34, 41) other than a transient, unique, localized renal vasoconstriction (30, 32), and no histological evidence of glomerular damage has been observed in a great mass of material examined (4, 10, 23).

The only evidence of glomerular injury derived from the many physiological studies of hemoglobinuria found in the literature is the simultaneous occurrence of albuminuria. However, the reports of this phenomenon are extremely inconsistent. In some experiments no albuminuria has been encountered, in many there have been slight traces only, while in a few, fairly large quantities have been observed. Marked variations have also been noted in the duration of the albuminuria, from short intervals at the height of hemoglobin excretion to periods lasting for several hours after the cessation of hemoglobinuria (12, 18, 35). The above findings and the relatively small and variable quantities of excess urinary protein encountered in this laboratory in dogs with induced hemoglobinuria are in very marked contrast to the regularity with which hemoglobin is excreted. In dogs and presumably other mammals the rate of hemoglobin excretion above a threshold level is directly proportional to its concentration in the plasma. It is difficult to conceive of any injury phenomenon initiating a mechanism responsible for such a relationship, which has been studied for plasma concentrations up to 1300 mgm. per 100 cc., and which is independent of the initial level of hemoglobinemia induced (32). If, on the other hand, hemoglobin circulating freely in the plasma increases glomerular permeability, it does so in direct proportion to its concentration above a critical level.

The nature of the glomerular membrane must be taken into careful consideration in any speculation about the manner in which a large molecule like hemoglobin may normally pass through it. Anatomically, capillaries in general and those of the glomerulus in particular are considered as having a very thin continuous endothelial lining. The protoplasm of these cells forms the semi-permeable membrane through which water and dissolved electrolytes may diffuse readily, and glomerular filtration pressure is equal to the difference between the hydrostatic pressure of the blood and the colloidal osmotic pressure of the plasma proteins.

Krogh (25) states that substances pass directly through the protoplasm of endothelial cells and also refers to pores in the walls of capillaries, although any mention of whether these are anatomical entities is carefully avoided.

There is apparently a relationship between the diffusion rates of

colloids and their passage through the walls of capillaries; however, it may be argued that this is just a coincidence and that in reality the escape of protein is restricted by the size of the pores in the membrane. The question of increased capillary permeability also presents a serious problem for although many causative factors are known, the nature of the underlying change is still undetermined.

Despite these uncertainties, the modern filtration theory of renal function, for all practical purposes, considers the glomerulus as a purely mechanical sieve containing innumerable pores each large enough to permit the passage of an inulin molecule (molecular weight 15,000). Such a concept indicates only a minimum but not a maximum pore size. The upper limit may be set at a molecular size approximately that of hemoglobin since no substances of higher molecular weight are known to pass through the glomerulus under normal conditions. The presence of a small amount of protein in fluid pipetted from the glomerular capsules of frogs suggested to Richards and Walker (38) that there might be a few pores in the glomerular membrane large enough to allow whole plasma to pass through and an expansion of this idea provides a clue to the possible mechanism of hemoglobin filtration. If actual pores do exist in the glomerular filter, it does not seem necessary to assume that all are of the same size and in fact studies on the excretion of hemoglobin and other proteins of smaller molecular size strongly suggest that gradations do exist. Muscle hemoglobin with a molecular weight of 17,500 (44) is excreted rapidly and has a clearance ratio with creatinine of about 0.58 in the dog (46). Since there is evidence of some tubular reabsorption of this substance its filtration rate has been estimated as about 0.75 times that of creatinine (46). Bayliss, Kerridge and Russell (4) studied the excretion of Bence Jones protein and egg albumin, proteins with estimated molecular weights of about 35,000, from perfused kidneys. No quantitative relative filtration rates are given but the authors state that they are only slightly below that of creatinine. Hemoglobin, at the upper limit, has an estimated filtration ratio with creatinine of only about 0.03 (32). This reflects the very low rate of hemoglobin excretion in relation to its concentration in the plasma and indicates that under maximal conditions only about 3 per cent of the pores in the membrane are large enough to allow a hemoglobin molecule to pass through. This pore size may not be an entirely physical attribute, but one modified by its electro-chemical charge and that of the colloidal molecules in contact with it. Pointing to this possibility are the reversible effects of such surface active sub-

stances as saponin (39) and of variations in pH (45) in modifying glomerular permeability to proteins.

The existence of a renal threshold for hemoglobin was disregarded by most of the earlier workers in the field and even in recent times few who have observed it have hazarded any guess regarding the mechanism involved. It has been implied (26, 35) that the escape of hemoglobin across the glomerulus does not occur unless a certain concentration is present in the plasma and conversely that, after the initiation of a high plasma hemoglobin level, 'passage of hemoglobin through the glomerulus ceases when hemoglobin ceases to appear in the urine. The dynamic activity which would be required to produce such a "glomerular threshold" is certainly not in harmony with the modern theories of glomerular filtration, for which there is an impressive mass of evidence. Insofar as the excretion of electrolytes is concerned the rôle of the glomerulus is a passive mechanical one, whereas the activity of the tubular epithelium is very highly specialized and selective. This tubular activity is entirely responsible for the renal thresholds of electrolytes so that the assumption of an entirely different mechanism for hemoglobin must be carefully considered, particularly since some type of tubular reabsorption is known to occur. The concept of a "glomerular threshold" was based largely on the ability to lower the initial threshold by about 46 per cent to a fairly constant level by repeated *diminishing* daily injections (26), and the failure to find deposits of iron-staining material in the epithelium of the convoluted tubules following repeated subthreshold doses. The lowered threshold was explained by assuming that the tubular epithelial cells became filled up to such an extent that they would reabsorb no more. It has since been shown (47) that the renal threshold for hemoglobin in relation to plasma concentration may be lowered by 60 per cent or more depending on the size and number of daily injections sufficient to cause gross hemoglobinuria. Since after lowering the threshold to various levels, the excretion rate curves are parallel to those obtained initially, and since under these conditions the estimated tubular reabsorption rate is lowered proportionally, it is not unreasonable to suppose that a complete cessation of tubular reabsorption would entirely eliminate the threshold. The constant level attained with diminishing daily doses in the earlier experiments suggests a state of equilibrium between the amount of hemoglobin reabsorbed by and the amount of hemoglobin products removed from the tubular epithelium daily, rather than a complete cessation of reabsorption. If modification of tubular reabsorption accounts for the lower threshold, the amount

removed daily is apparently quite significant, for when injections of hemoglobin are discontinued, it returns to its initial level within a period of several weeks (26). Such a mechanism could also explain the absence of iron-staining pigment in the kidney following repeated subthreshold injections since the amount picked up would be less than the rate of daily removal from the tubular epithelium.

In this connection it is important that the curves relating the clearance ratios of hemoglobin and creatinine to the plasma concentration of hemoglobin are almost identical in form, though not of course in magnitude, with those for other threshold substances such as glucose (32). This indicates at least a similarity in excretory pattern which implies for hemoglobin a constant though small rate of glomerular filtration, proceeding until the plasma concentration reaches zero, and a rate of tubular reabsorption which at the threshold is at a maximum and equals the amount of hemoglobin escaping into the tubular lumina.

Figure 1 illustrates the type of graphical analysis of experimental data by which quantitative estimates of the glomerular filtration and tubular reabsorption rates of hemoglobin have been made (32). Line *A* represents the actually determined relationship between excretion rate, in terms of milligrams per minute, and the plasma concentration. The parallel line *B* originating at zero is taken to represent the glomerular filtration rate in relation to the plasma concentration if tubular reabsorption, the perpendicular distance between lines *A* and *B*, reaches a maximum at the threshold and remains constant at higher levels.

An average value for the initial rate of tubular reabsorption lies between 2.0 and 3.0 mgm. per minute (32), and values of less than 1.0 mgm. per minute have been obtained after lowering the threshold by about 70 per cent (47).

The data presented by Bogniard and Whipple (6) show clearly that at least some of the iron-containing portions of hemoglobin are stored in the tubular epithelium. They found the iron content of the kidney markedly elevated after multiple intravenous injections of hemoglobin, in amounts sufficient to cause hemoglobinuria. Dense iron-staining deposits of pigment were seen in the convoluted tubular epithelium many weeks after cessation of such injections when the animals were kept in a normal condition, but the pigment disappeared promptly when anemia was produced by bleeding. More recently iron retention by the kidneys has been determined following the injection of hemoglobin containing radioactive iron (47). Single large injections sufficient to initiate plasma concentrations of about 700 mgm. per 100 cc. were given

to dogs that had not previously received any hemoglobin and to those in which the threshold had been markedly lowered by repeated injections. Tubular reabsorption values were estimated from the rate of pick-up obtained by the graphical analysis outlined above and the time required for the disappearance of hemoglobinemia. The animals were subjected to viviperfusion twenty-four hours later and comparisons were made between the above estimates and the actual amounts of radioactive iron

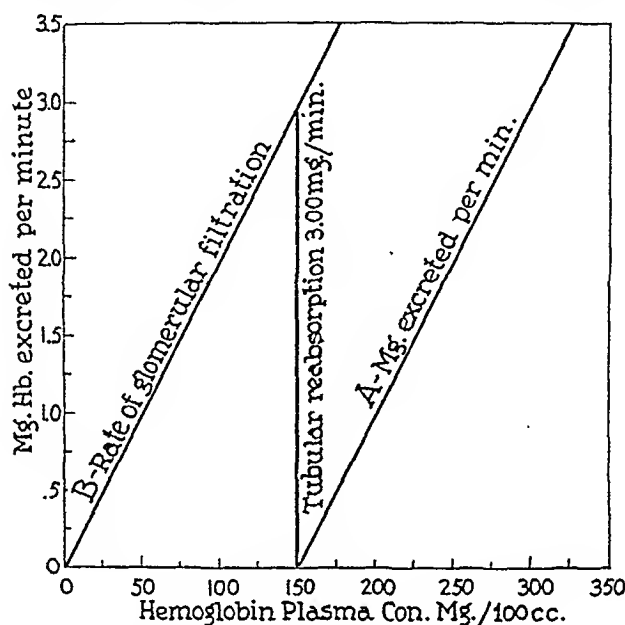


Fig. 1. Graph illustrating the type of analysis of experimental data used to estimate tubular reabsorption. Line A represents the actually determined rate of renal hemoglobin excretion above the threshold. The parallel line B equals the rate of glomerular filtration if tubular reabsorption, the perpendicular distance between the two lines, reaches a maximum at the threshold and remains constant at higher levels.

found in the kidneys. Calculated values ranged from somewhat over 40 per cent of the injected amount in the normal group to about 20 per cent in the group with thresholds previously lowered by repeated hemoglobin injections. However, only about 16 per cent of the injected iron was present in the kidneys of the normal animals contrasted with over 20 per cent in the kidneys of comparable animals with lowered thresholds. These results cast some doubt on the interpretations originally presented by Monke and Yuile (32), which hold true only if it

is assumed that in normal animals there is a very rapid removal of hemoglobin or its breakdown products from the kidney after it is picked up from the tubular lumina. It must also be assumed that both the intake and output of hemoglobin by the cells of the convoluted tubules are greatly diminished, following repeated injections daily, since under these conditions the amount of iron in the kidneys twenty-four hours after injection equals the total estimate of tubular reabsorption. If the low radioactive iron content of the kidneys in the group of normal animals represents the *total* amount reabsorbed during the entire period of hemoglobinemia, it seems necessary to assume that the glomerulus is responsible, at least in part, for the threshold phenomenon and that it is the permeability of this structure rather than reabsorption by the tubular epithelium which is modified by repeated injections. At the present time much of the evidence favors the theory that tubular reabsorption alone is responsible for the threshold phenomenon but the final proof must await the outcome of further investigations.

Many investigators have estimated the renal threshold level for hemoglobin in man and various animals, but comparisons are often difficult to make. Although a renal threshold for any substance is essentially a function of its plasma concentration, most hemoglobin thresholds have been given as the minimum amount of injected hemoglobin, per kilo of body weight, required to cause hemoglobinuria. This of course will be approximately parallel to the plasma concentration but does not take into consideration variations in plasma volume due to differences in such factors as the state of nutrition or hydration, nor the speed at which the material is injected. Values ranging from 60 mgm. per kilo to about 400 mgm. per kilo have been reported (26, 29, 35, 36, 41). Recent estimations based on more accurate urinary determinations correlated with the plasma concentration of hemoglobin, in a large series of dogs, were found to vary as a rule from about 80 to 150 mgm. per 100 cc. of plasma (32), while in a small group of humans with experimentally induced hemoglobinuria the threshold level was found to lie at about 135 mgm. per 100 cc. (18).

The nature of the process by which hemoglobin, having passed through the glomerulus, is picked up by the epithelium of the convoluted tubules, is apparently quite different from that applying to the movement of glucose and like substances which pass readily from the tubular lumina back into the blood stream.

As previously stated, the tubular epithelial cells exhibit a process of storage and disintegration of the hemoglobin molecule which is not un-

like that seen in the liver, spleen and lymph nodes. Gerard and Cordier (16), in an extensive and careful series of experiments, have studied the mechanism by which various colloidal substances, including hemoglobin, are picked up and stored by the convoluted tubules of the toad (*bufo vulgaris*, *bufo calamita*). Their results lend considerable support to the mammalian findings previously outlined. The term "athrocytosis" has been applied to the process by which the tubular epithelial cells form storage granules from ingested colloidal particles. This is a most pronounced phenomenon when studied in the open nephron of the toad. By way of this open nephron, colloidal aggregates of any size can be readily transferred to the unmolested tubular lumen. Microscopical observation reveals that the striated border cells of the proximal segment ingest colloids presented at their apical poles. The cells do so in terms of a molecular size gradient, athrocytosing the smaller colloids in the more proximal portions of the tubule. In the closed nephron this phenomenon is limited to those molecules of near colloidal size which can normally or pathologically pass across the glomerular membrane.

It is interesting to speculate on the manner in which these large colloidal molecules pass into epithelial cells such as those lining the convoluted tubules. Comparison is difficult with the ameoboid activity responsible for the phagocytosis of foreign particles by freely wandering histiocytes but the active ingestion of colloidal and particulate matter by the fixed cells lining the sinusoids of the spleen, lymph nodes and liver is perhaps more analogous. In this connection it may be significant that tubular reabsorption of colloids occurs only in that part of the nephron which is mesodermal in origin. This specific problem is linked to the broader one of the passage of large aggregates across cell membranes and the answer may possibly be found in some enzyme activity on the cell surfaces.

Newman and Whipple (33) submit the theory that, in the dog, conservation of hemoglobin is the underlying physiological purpose of tubular reabsorption. The ontogenetic and comparative anatomical studies of Gerard and Croder (16) justify the application of the same theory to the phenomenon of athrocytosis. The celomic fluid of primitive forms contains quantities of protein solutes, the elimination of which by the open nephron would be a definite loss to the organism. In these lower forms the striated brush border segment functions as an organ of recovery which has become largely vestigial, with the development of a closed nephron.

Although it was originally stated by Drabkin, Widerman and Landow

(13) that about 10 per cent of injected hemoglobin is excreted in the urine, this has been shown to be true only for injections of 100 to 150 mgm. per kilo. Above this level the percentage recovery in the urine increases to as high as 35 to 40 per cent, approximately in proportion to the amount injected (18, 32). It is interesting to note that following the injection of large quantities of hemoglobin much more is removed by renal excretion and retention than was previously thought to be the case.

The foregoing concepts of renal hemoglobin excretion are largely based on quantitative studies in which the urine was rendered and kept alkaline. Under these conditions hemoglobin remains completely in solution during its passage through the kidney and after reaching the bladder. In the presence of acid urine, on the other hand, precipitation within the renal tubules readily occurs causing the formation of casts. The obstruction due to this phenomenon has been thought to be responsible, at least in part, for the fatal anuria which frequently follows transfusions of incompatible blood. However, in view of reports of fatal transfusion reactions with anuria in which little or no hemoglobin was found in the form of casts in the kidneys (11, 12) and the absence of any untoward effects in experimental animals with either acid or alkaline urine from hemoglobinuria induced by the injection of carefully prepared, stroma-free hemoglobin, some discussion of this controversial subject is warranted.

In 1925 Baker and Dodds (3) produced a marked retention of non-protein nitrogen in the blood of rabbits with acid urine by the intravenous injection of hemoglobin but noted no such disturbance when the urine was alkaline. More recently DeNavasquez (12) has injected large doses of hemoglobin into groups of rabbits with both acid and alkaline urine. Some rise in blood urea occurred in either case, but no apparent disturbance in renal function was noted, as judged by phenol red clearance. However, there were marked differences in hemoglobin excretion. Thirty per cent of the injected hemoglobin was recovered in the urine of the acid group as contrasted with only 12 per cent in the alkaline group. On the other hand, the kidneys of the group with alkaline urine retained about 50 per cent more hemoglobin than those of the group in which the urine was acid. Such variations apparently depend on the state of solution of the hemoglobin and the resulting differences in tubular reabsorption.

The carefully controlled experiments of DeGowin, Osterhagen and Andersch (10) show conclusively that although a high percentage of

dogs with strongly acid urine will succumb to transfusions of large amounts of laked red blood cells, death does not occur following similar injections when the urine is alkaline. These experiments do not prove that the precipitation of hemoglobin in the kidney is responsible per se for the observed disturbances, despite the fact that the most striking histological difference between the two groups of animals is the presence of numerous hemoglobin casts in the renal tubules of those with acid urine. Since severe (7) and even fatal (12) transfusion reactions can occur when the urine has been previously alkalized and remains so throughout, some additional unknown factor, as suggested by DeGowin (11) must be present. Following the transfusion of incompatible blood it is possible that the *foreign protein* of the donor's stroma is responsible for the initial anaphylactoid reaction encountered and the destructive tubular lesions frequently seen in the kidneys. However, this would hardly hold true for the occasional cases of renal failure associated with black water fever and hemoglobinuria due to various drugs. In any event, it is reasonable to assume that the additional-precipitation of a large amount of hemoglobin in the tubular lumina will secondarily increase the severity of any reaction.

Since in other types of clinical hemoglobinuria the problems are related to the underlying hemoglobinemia rather than to renal function, they will not be discussed at this time.

Despite the fact that subsequent investigation may modify some of the theoretical concepts here presented, the realization within recent years that hemoglobinuria is a well regulated and reproducible phenomenon should help to clarify much of the earlier confusion relating to this subject.

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THE FUEL FOR MUSCULAR EXERCISE

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There is perhaps no animal texture as to the nature of which more contrary opinions have been held, or more conflicting statements advanced, than that of voluntary muscle, so that even at the present time it must still be considered a question by no means set at rest. (Dobie, 1849.)

The type of metabolites used during muscular contraction has attracted the attention of biochemists and of physiologists for many years. This widespread interest in the problem arises not only from the theoretical implications of this matter but also from its practical application. In spite of the almost universal use of machines, muscular energy developed by man is still a fundamental necessity. It is, therefore, of importance to determine the type of food material used in muscular exercise, the ratio of conversion of energy to useful work provided by various diets, and many other related problems. The limitations of space necessitate exclusion from this review of many interesting problems. Only the following will be discussed: the effect of diet on muscular efficiency and prolonged muscular exertion, the respiratory quotient during exercise, the changes in blood sugar and in blood lactate, and finally, the output of nitrogen in the urine during and after exercise. This discussion will further be limited to experiments done for the most part on man, only an occasional reference being made to results obtained on other animals. No detailed references will be made to the utilization of fat by muscle because this subject has recently been reviewed by Gemmill (1940).

Mechanical efficiency. The mechanical efficiency of men and of animals has been used to determine the character of metabolism during muscular exercise. To the speed and the load, the two chief variables that affect efficiency of muscular work, a third variable may be added, namely, the effect of previous diet on the efficiency. If the different food materials are used in isodynamic equivalents, there will be no change in

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efficiency for either the oxidation of carbohydrate, fat, or protein during the exercise. However, if the body must convert one form into another before it can be used to supply energy to the contracting muscles, there may be loss of energy in this conversion and the mechanical efficiency may be lowered. There have been numerous calculations for the determination of this loss, especially for the conversion of fat to carbohydrate. The calculated results range from endothermic "loss" to exothermic "gain." The various equations postulated for the interconversion of fat to carbohydrate and their thermodynamic possibilities have been reviewed by Rapport (1930). It is obvious that none of the theoretical thermodynamic calculations have any meaning until the exact chemical pathways and the total amounts of material entering into the interchange are known.

Two general methods have been used in this type of experiments. In the first, the subject is given a diet high in one of the primary food materials and low in the others. After several days of such feeding, the subject exercises in the basal condition. The object of these experiments is to saturate the body with one foodstuff and to deplete it of the others. The difficulty of interpreting the results is that food materials are converted into different forms in the body. Proteins, for example, are readily converted to carbohydrate, and carbohydrates are changed to fat.

The second method used in this field is to superimpose exercise on to the specific dynamic action of the food material in question. These experiments are made with the subject exercising after taking a meal rich in the food material under study. These studies are based on the theory that the ingested food material will be used during exercise, an idea not founded on any experimental proof. One of the difficulties in deciding whether there is a difference in efficiency in the latter experiments depends on the base line used in these experiments. The extra metabolism for work is superimposed on the extra metabolism which is due to the ingestion of protein (Anderson and Lusk, 1917; Rapport, 1929), but not on the specific dynamic action of carbohydrate or of fat. This means that in the case of a protein meal the gross efficiency based on the total energy expended may be less after the meal than under basal conditions, whereas the net efficiency based on the extra metabolic activity above the specific dynamic activity may not change. On the other hand, as compared to basal conditions, the gross efficiency may not change when exercise follows a fat or carbohydrate meal, but the net efficiency calculated from the difference between the metabolism during exercise and

just before exercise would give an erroneous idea of the rise in metabolism which was due to the exercise itself. Therefore, in experiments of this type, the best efficiency to use for exercise following a protein meal would be net efficiency; the best following a fat or carbohydrate meal would be the gross efficiency. Although there are many additional difficulties, both theoretical and experimental, in using the mechanical efficiency of man for the determination of the type of food material used by the muscles in exercise, the findings of such experiments are of interest. They demonstrate clearly that there is very little change of muscular efficiency in a subject fed on diets of carbohydrate, fats, or proteins.

There have been numerous determinations of muscular efficiencies made on man in a basal state following diets high in fats, carbohydrates, or proteins. Benedict and Cathcart (1913) report values of 5.37 Calories produced for each Calorie of external work following a carbohydrate rich diet, and 5.31 Calories on a carbohydrate poor diet. Krogh and Lindhard (1920) reported their results in terms of net energy expenditure per unit of work for theoretically determined respiratory quotients. By projecting a straight line through the experimentally determined points, they found the theoretical energy expenditure for respiratory quotients of 1.00 and 0.70. The difference between those theoretical values gave their subjects an average of 11 per cent greater efficiency on a carbohydrate diet than on a fat diet. However, in some of their series it is obvious that there is not a straight line relationship between these two variables (for example, on p. 328, fig. 15); and, therefore, the projection of a straight line through the determined points may give erroneous theoretical values for the Calories used at the respiratory quotients of 0.7 and 1.00. There can be no doubt that their subjects were slightly more efficient on carbohydrate diets, but the order of magnitude may be less than 11 per cent depending on how the line is projected to the ordinates. The work of Krogh and Lindhard was continued in their laboratory by Bierring (1932). His results also leave no doubt that the Calories expended are higher at the lower respiratory quotients, but, again, like Krogh and Lindhard, this author uses a projected line to obtain the two extremes for his calculation. Taking the projected values at respiratory quotients of 1.0 and 0.70, he claims that there is an 8.3 per cent difference in efficiency when fat is used as compared to carbohydrate. As actually determined (fig. 6, p. 39), the highest R.Q. obtained was 0.93 where the work expenditure was 4.6 Calories; the lowest R.Q. was 0.75 where the Calorie output was 4.82. Therefore, the actual difference is 4.5 per cent between these two determined points.

Reynolds, Sevringhaus and Stark (1927) attempted to repeat the ex-

periments of Krogh and Lindhard. Their subjects were not in basal condition as the experiments were conducted 1.5 hours after the noon meal and the subject's only rest before the experiment consisted of sitting 12 to 15 minutes on the ergometer. Records of oxygen consumption were taken from a spirometer which gave oxygen consumption for periods of less than two minutes. Since respiratory quotients were not determined, it is impossible to analyze their results in the same manner as those of Krogh and Lindhard. In some of the experiments the work was very light, for example, in subject M. S. R. only increasing the oxygen consumption to 1254 ml. per min. No attempt was made to keep the work constant as can be seen by the variation in oxygen consumption during work. For example, in subject E. L. S., the oxygen consumption varied from 883 ml. per min. to 2411 ml. per min. They concluded from this experiment that the average net efficiencies on mixed carbohydrate or fat diets did not vary. In view of the conditions under which these experiments were made, their results will not be given any weight in the summary. Marsh and Murlin (1928) have reported net efficiencies of a subject given a normal diet, a high carbohydrate diet, and a high fat diet. The work was very light, only raising the oxygen consumption to 668 ml. per minute. They observed that the net efficiencies on the three diets were about the same, but on continuing the diets there was a decrease in efficiency on the fat diet, especially noticeable after the third or fourth day. The difference between the efficiencies on the carbohydrate and fat diets was of the same order of magnitude as reported by Krogh and Lindhard. Wishart (1934) fed a professional cyclist diets containing varying amount of proteins and found a higher gross efficiency on the lower protein diet, although the subject could pedal for a longer period of time on the higher than the lower protein diet. Even when a correction was made for the specific dynamic action of the protein, the difference between the efficiencies was still clear. In very carefully controlled experiments Cathcart and Burnett (1926) reported a slight decrease in oxygen consumption per kilogram meter of work when the subject was on a carbohydrate diet. The value obtained with the subject on a fat diet was comparable to that observed with the subject on a mixed diet. The most recent experiments in this field are those of Christensen and Hansen (1939) in which the results were obtained under conditions of strenuous muscular exercise. In one individual the net energy production was lower at a R.Q. of 0.78 (11.50 Cal. per min.) than at a R.Q. of 0.92 (11.90 Cal. per min.), but in the other two subjects there was a slight increase in the output of energy on fat diets.

The second method of experimentation in this field, that of feeding

certain food materials before the exercise, has given the same results as the experiments described above in which the experimental subject was in the basal condition after a diet high in one food material. The first thorough study in this field on animals was made by Anderson and Lusk (1917). These authors measured the resting metabolism of a dog under basal conditions and compared it with the excess metabolism of the dog running at a definite rate soon after the ingestion of glucose, meat, or alanine. This type of experimentation does not give the mechanical efficiency of exercise in the true physical sense, but does give comparable values for energy output for performing a definite amount of work. These workers report that it requires 0.580 kilogram meter of energy to move one kilogram of the body weight of a dog one meter with the dog under basal conditions, after a long period of fasting and after meat or alanine ingestion. There was a slight decrease in this value when carbohydrate was given. Following ingestion of 70 grams of glucose, 0.579 and 0.555 kilogram meter per kilogram were expended in two experiments. After taking 100 grams of carbohydrate, a value of 0.550 was obtained. These results demonstrate that the energy expended for running in a dog on a carbohydrate diet is slightly less (5 per cent) than during starvation or on a protein diet. Rapport (1929) extended the observations of Anderson and Lusk by determining the metabolism of running after the ingestion of fat as well as carbohydrate and protein. He found that 2.40 Calories were expended to move 1 kilo of body weight of the dog one meter under basal conditions, 2.38 Calories after ingestion of glucose, 2.54 Calories after the ingestion of fat. These results demonstrate that the energy expenditure after a carbohydrate diet was the same as during the post-absorptive state and was slightly greater after fat ingestion. Rapport also confirmed Anderson and Lusk in their observation that the metabolism during exercise was superimposed on the specific dynamic action of protein.

In man, there have been numerous studies of the effect of ingestion of food on mechanical efficiency. Carpenter and Fox (1931) could not detect any change in net efficiency from the basal level following the ingestion of glucose and fructose when the subject was working at the rate of 275 and 555 kilogram meters per minute. In contrast, Haggard and Greenberg (1935) in their monograph on Diet and Physical Efficiency, give very remarkable changes in the net efficiency of man following a meal. In one individual the efficiency rose from 19.5 per cent to 27 per cent following breakfast. By 12:00 noon it had fallen to 21.2 per cent, but on taking the noon meal it rose to 26.0 per cent. Since eating fat

in the form of olive oil did not change the efficiency, and eating lean beef or glucose did, these authors concluded that protein and carbohydrate in the diet had the ability to increase the efficiency. These experiments were made on a bicycle ergometer, on which the subject pedalled for five minutes. A collection of expired air was made for that period and for a ten minute recovery. The work was light, raising the oxygen consumption to 1500 ml. of oxygen per minute.

These experiments are at variance with others performed in this field. Repetition of these experiments by Haldi, Bachmann, Ensor and Wynn (1938) failed to show any difference in efficiency following the ingestion of glucose, or after breakfast, although there was a rise in the respiratory quotient. Carpenter and Lee (1938) also determined net efficiencies after the ingestion of sucrose and galactose and found only a small change in efficiency. The control experiments, in which 275 kpm. of work per minute was performed, gave values varying from 18.4 to 20.2 per cent; experiments with sucrose gave values ranging from 17 to 20 per cent, but those with galactose showed slightly higher values, 19 to 21 per cent. Increasing the work to 550 kilogram meters per minute after the subject had ingested various sugars, gave values for efficiency higher than in the preceding series. The range was now from 21 to 26 per cent. However, the basal values for the efficiency of these experiments were higher, ranging from 21.9 to 24.7. These experiments show that the ingestion of food material does not change the efficiency of muscular exercise. In light of this work and that of Haldi and his associates, it is impossible to accept the conclusions given by Haggard and Greenberg. The general assumption must be made that ingestion of food material has very little effect on efficiency of muscular exercise.

The conclusion to be drawn from the experiments on the determination of muscular efficiency following various diets is that the efficiency is practically the same on all diets. There is a slight increase in efficiency following a high carbohydrate diet, but probably not more than 5 per cent.

Duration of exercise on different diets. Another approach to the problem has been the determination of the working capacity of man and animals on different diets. In these experiments the end point used is fatigue. In dogs Dill, Edwards and Talbott (1932) have shown that a dog can run 17 hours without exhaustion when given carbohydrate, but without food the dog at its best could do only $4\frac{1}{2}$ hours. By giving carbohydrate to a fatigued dog, the animal was revived and was able to resume work. Unfortunately, in these experiments, other food mate-

rials were not given. The most recent experiments on man in this field are those reported by Christensen and Hansen (1939). Working with an intensity of 1080 kgm. per minute, their subject was able to continue three times as long on a carbohydrate diet as on a fat diet.

Recently it has been claimed that the ingestion of gelatin will enable a man to combat fatigue. Ray, Johnson and Taylor (1939) reported that the taking of 60 grams of gelatin a day increased the length of time that men could ride a stationary bicycle before fatigue set in. In ten subjects, 6 men and 4 women, remarkable increases in work capacity were observed in the men on the gelatin diet but not in the women. Hellebrandt, Rork and Brogdon (1940) repeated this work on a group of women accustomed to severe physical activity. They exercised on a bicycle at such a rate that fatigue set in after 40 seconds of work. The taking of gelatin did not aid in prolonging the work capacity under these conditions. It is doubtful whether the taking of any food material will aid in anaerobic work but only in the maintenance of moderate work for long periods of time. Maisson (1940) studied the work capacity for contraction of the extensor digitorum communis before, during and after the ingestion of gelatin and aminoacetic acid. After one year of muscular training, neither of these two substances aided in increasing the working ability of this muscle. This author points out that Ray's subjects were incompletely trained. Robinson and Harmon (1941) have reported recently their studies on the effect of gelatin on training to exhausting work. They found that the ingestion of gelatin had no effect on the oxygen debt nor the speed of running. These experiments should be repeated with determinations similar to those of Wishart who found a prolongation of working capacity on high protein diets. Gelatin may aid prolonged muscular activity indirectly by carbohydrate formation.

Atzler and Lehmann (1937) claimed an increase in the output of work during a diet high in lecithin. The ability for both static and dynamic work increased.

The conclusion drawn from these experiments is that carbohydrate is necessary for long continued muscular work. There is not sufficient evidence at the present time to state that other substances such as gelatin, amino acids or lecithin also have this property.

Respiratory quotient. Another popular approach to this problem has been the determination of the respiratory quotient during work. Although difficult to determine, especially during severe exercise, and difficult to interpret, nevertheless, many determinations have been made of this much abused quotient. Carpenter (1931), in his review of the work

up to 1931, concluded that during light work the respiratory quotient does not change, but with heavier work the respiratory quotient rises and as work is continued, falls. He interpreted these changes as indicating that the same mixture of metabolites is burnt during light muscular work as during rest, that heavy work increased the proportion of carbohydrate used, and that long-continued work diminished the supply of carbohydrate and accentuated the utilization of fat. This problem was reviewed also by Dill (1936) with conclusions similar to those by Carpenter.

The only disturbing factor in these general conclusions is the high respiratory quotients reported by Best, Furusawa and Ridout (1929). These authors reported quotients during mild exercise equal to the basal quotients, reaching unity for moderate exercise, and quotients above unity for strenuous work of short duration. It was obvious from their report that in the experiments after strenuous work complete recoveries were not obtained following the exercise, for recovery periods of only 20 to 50 minutes were used. Even in these determinations the R.Q. for the total metabolism was rarely above 1.00, although values for the excess metabolism as high as 1.65 were reported. These experiments were repeated by Gemmill (1931) using adequate controls. The subjects were first trained to breathe through valves under basal conditions for three hours in order to accustom them to the procedure. In order to obtain basal samples, they slept over night in a comfortable bed beside a treadmill. Following this, they ran for ten seconds on the treadmill at a fast rate and then returned to the bed after the exercise. Thus, the only variable in the experiment was the exercise. Collections were made for three hours after the exercise. In these experiments, the gross respiratory quotients were less than one, and the excess respiratory quotients for the three hour period were 1.10, 0.86, 0.85 and 1.00. This work was again repeated by Solandt and Ridout (1933) but they did not use the extreme precautions observed by Gemmill for obtaining basal conditions. In some of their calculations, these authors used the normal variation in basal metabolism as the basis for a complete recovery period; i.e., when the oxygen consumption and carbon dioxide production returned to these limits it was considered complete. In one subject, the oxygen consumption and carbon dioxide production returned to these limits in one and one-half hours; in the other two, there was a slight increase in oxygen consumption over the basal even after one and one-half hours of recovery. They dismiss this portion of the recovery period as not being due to recovery and claim that recovery is complete in one and one-half hours

after the exercise. Under these conditions the excess R.Q. is above unity. This type of argumentation reduces the excess respiratory quotient to one of definition. If this quotient is defined as the ratio of carbon dioxide produced above the basal control experiments to the oxygen used above similar basal control experiments, one quotient is obtained. If the basal control experiments are used for the carbon dioxide calculation and the post exercise base line (table VI, Solandt and Ridout, 1933) for the oxygen consumption, another "excess quotient" is obtained. It is interesting to note that using the first method several of the excess quotients reported by Solandt and Ridout (table V) are approximately unity for the two and one-half hours' recovery.

Two additional papers have appeared showing that the quotient is less than 1.00 for severe muscular exercise. Dill and his associates (1937) reported quotients between 0.9 to 1.0 when their results were corrected for the shift in carbon dioxide tension at the end of a two hour recovery period. Christensen and Hansen (1939) reported gross quotients of 0.95 with their subjects working at a rate demanding 4.15 liters per minute.

The work of Brand and Krogh (1935) is of interest in this connection. They observed a lowering of the respiratory quotient in rats after a working period, and with this low respiratory quotient there was a new formation of carbohydrate.

The conclusions from this work are that the gross respiratory quotient for muscular work is unity or less than unity. The "excess" respiratory quotient obtained by using the basal level before exercise as a base line is also of that order of magnitude. When other base lines are used such as the post exercise oxygen consumption at an arbitrary time of one and one-half hours after exercise, the "excess" respiratory quotient may be above unity. Since the "excess" respiratory quotient depends on a definition of a base line, this conclusion alone warrants its dismissal. Therefore, the conclusions reached by Carpenter in 1931 still hold for the metabolic events occurring in muscular exercise as revealed by the respiratory quotient determinations. In surveying the work done on the respiratory quotient, the reviewer has reached the additional conclusion that its determination during muscular work has not been worth the time, trouble, and effort put on this phase of the study.

Chemical changes in blood. Another general approach to the problem of the fuel for muscular exercise has been the determination of the changes in concentrations of substances in the blood before, during and after exercise. The concentration of any metabolic substances in the

blood is the resultant of many variables. The main factors are the supply of the substance and its utilization. The latter, in turn, depends upon oxidation and deposition. However, many additional factors besides supply and utilization may change blood composition during exercise. Exercise may cause the blood to become more concentrated, and this, in turn, will affect the concentration of all substances in the blood. The rate of blood flow increases, a phenomenon which changes the rate at which chemical substances reach the tissues. The pH of the blood may change, thus affecting the ratio of ionic concentrations in the blood and causing retention or elimination of base by the kidney. The kidney threshold for blood constituents may change. Capillary permeability may vary in exercise. Therefore, for a change in blood concentration to be significant, it must be very marked. Even when the change is of such magnitude that there is no doubt that it was produced directly by muscular contraction, exact interpretation of the change is difficult. The two following examples, the effect of exercise on the glucose and on the lactate concentrations in the blood, will illustrate these difficulties.

Blood glucose. There have been many studies of the effect of exercise on the glucose level in the blood. These findings fall into three groups: under certain conditions the blood glucose level increases; under others, it remains constant; and under still other conditions of exercise, it falls. An analysis will be given of these results.

Edwards, Richards and Dill (1931) examined the blood glucose of a group of football players before and after a game. Before the game the players and substitutes had normal blood sugar levels, but in the game only the players had elevated glucose levels. In these same players, carrying out exercise under laboratory conditions, there was a decrease in blood sugar. The authors of this paper claim that the elevation was an emotional rise. It is difficult to accept this explanation. The players on the bench are under an emotional strain as well as the men in the game. Nevertheless, their blood sugar levels did not change. In some of the players, the blood sugar level was 180 mgm. per cent fifty minutes after the game was over. This could hardly be a continuation of an "emotional" effect. Other factors besides emotion must play a rôle in this continued rise in blood sugar. Solandt and Ferguson (1932) also report an elevation in the blood glucose level persisting thirty minutes after the subject had run a mile in 5 to 6 minutes. In one subject, the resting level before the race was 119 mgm. per cent, but 30 minutes after the exercise it was 190 mgm. per cent. Dill, Edwards and Mead (1935) reported an increase in blood sugar lasting for eighty minutes in an in-

dividual after working. Thornton and White (1933) give a value of 191 mgm. per cent glucose in a member of the 1933 Cambridge boat race crew 22 minutes after a race ended.

This long continued rise in blood sugar is of such definite character that it is of great interest. Several factors promote the continuous rise: 1, severe but not exhaustive exercise; 2, intermittent exercise; 3, lactate formation. As stated above, the psychological effect of emotion may be omitted as a causative factor. The work of Brouha, Cannon and Dill (1936), if it can be applied to man, rules out the sympathetic nervous system as a cause, for they demonstrated that the blood sugar varied within normal limits in the exercising sympathectomized dog. Since the high blood sugar levels accompany lactate formation, the cardio-vascular and respiratory systems must not be able to supply the muscles with sufficient oxygen during the exercise. The presence of lactate in the blood may be a stimulus for production of glucose from glycogen in the liver. As long as lactate is present, glucose will be formed from the glycogen stores after exercise as well as during exercise.

Light muscular exercise does not change the blood sugar level. Many workers have reported this finding. The most interesting recent papers in this connection are those of Douglas and his associates. They not only studied the blood sugar level, but also evaluated the carbohydrate reserves in the body by determining glucose tolerance curves and by observing the changes in blood sugar following the injection of adrenalin and insulin. In the first paper Courtice and Douglas (1936) reported that after the subject had walked 10 miles at the rate of 4.5 per hour, there was no change in his blood sugar level, but there was a slight decrease in the respiratory quotient. On ingestion of glucose, there was only a slight rise in respiratory quotient and a delay in the increase in blood sugar. The fall in this level was more prolonged after exercise than before. Mills (1938) has confirmed these changes in glucose tolerance after light muscular exercise. Margaria (1939) reports also a marked fall in the respiratory quotient following muscular work a decrease which was not associated with a marked change in blood sugar. These findings were confirmed and extended by Douglas and his associates (1939) in their second paper. The glucose tolerance curve after exercise rises to a greater height than before and was very similar to the curve obtained on a low carbohydrate-high fat diet. Injection of insulin after exercise showed no evidence of any change in sensitivity to this hormone. In a third paper Douglas and his associates studied (1939) the effect of adrenalin on the blood sugar of a subject after light

muscular work on a bicycle ergometer. The working period was from $2\frac{1}{2}$ to 3 hours. Comparison of these experiments with the resting experiments showed that the rise in blood sugar concentration following the injection of adrenalin is less and recovery more rapid during work than during rest. These experiments of Douglas and his associates demonstrate that the carbohydrate stores of the body are depleted although the blood sugar does not fall during light muscular exercise. Similar experiments should be carried out in individuals who have worked to a degree when their blood sugar level is actually lowered, in order to see how their carbohydrate stores would function under the action of insulin and adrenalin.

Long continued exercise decreases the blood glucose level. Levine, Gordon and Derick (1924) and B  st (1930) reported this result for runners following the Marathon. One interesting finding was that the men with the most marked signs of physical exhaustion had the lowest blood sugar levels. The blood sugar level also falls in men running for long periods on a treadmill (Edwards, Margaria and Dill, 1934).

Strandell (1934) has carried out a series of observations in which glucose was injected just before exercise. If the exercise was severe, the blood glucose did not change; however, during slight exercise, there was an increase in blood sugar. These changes were not due to deficient absorption of the glucose from the digestive tracts. These experiments also indicate that glucose is used by the contracting muscles under severe conditions.

The lowering of the blood glucose level during long continued exercise denotes a marked utilization of carbohydrate for muscular exercise. The rise during and after severe exercise of short duration indicates that the mechanism of supply overbalances the mechanism of utilization. Since the rise accompanies lactate formation, it is suggested that lactate may be the stimulus for the production of glucose from glycogen in the liver under these conditions. In moderate exercise of short duration, the level of blood glucose does not change. It may be assumed that the blood glucose is being used, but in these cases the supply meets the demand.

Lactate. Another substance which has been studied extensively in the blood during and after exercise is lactate. Although analyzed and reported as lactic acid, there is very little lactic acid in the blood, since at the normal pH of the blood this substance is in its ionized form, the lactate ion. Therefore, in this review, the term "lactate" will be used to describe this substance.

Although it was known for some time before the work of Hill and his associates that lactate was formed in the body of man during severe muscular exercise, it was through the efforts of these observers that a possible relationship between speed of working, lactate formation, and duration of recovery period was established. Hill and his associates (1923) postulated that there was a direct relationship between the oxygen used above the base line after exercise and the lactate concentration in the body. They assumed that all of the extra oxygen consumed during the recovery period was due to oxidation of lactate. Hill calculated that an oxygen debt of one liter represented in the body the presence of 8.1 grams of lactate at the end of exercise. Hill and his associates also assumed that even moderate effort increased the lactate concentration in the blood. Long (1926) offered an example of this theory in describing an increase in lactate in the blood of individuals walking at the rate of 3.3 miles per hour. Experimental work during the past ten years has modified these views. Owles (1930) described a critical metabolic level during exercise of varying degrees of severity. At moderate degrees of exercise, there was no increase in lactate in the blood; at more severe grades, lactate accumulated. A more detailed account of the relationship of severity of exercise to lactate production was published by Margaria, Edwards and Dill (1933). These authors described experiments in which blood was drawn from the femoral artery and vein as well as from an arm vein, at various times after exercise. The values for lactate concentration in these various samples were compared to the oxygen debt. The usual difficulty in determining exactly the duration of this debt was encountered, as is evident from their observation that the oxygen consumption in their individual was 30 ml. per minute above the basal level even two hours after work had stopped. This increment they disregarded in their determinations, and took instead an arbitrary value of one and one-half hours for their recovery periods. Their results show clearly that there was no lactate appearing in the blood for oxygen debts up to 3.0 liters per minute. Above this value, there was a straight line relationship between lactate concentration and oxygen debt. These authors conclude that there are two phases to the recovery period, an alactacid debt which does not depend on lactate production and a lactacid debt which is related to the oxidation of lactate. Cook and Hurst (1933), at the time of publication of Dill's work, showed that the exercise of walking at the rate of 4.5 miles per hour for 30 minutes did not change the concentration of lactate in the blood of the femoral vein. These authors also report the interesting comparison of lactate concen-

tration in arterial and venous bloods following running to complete fatigue in 1 minute. In two subjects from whose femoral arteries and veins simultaneous blood samples were taken 3 minutes after the exercise, the venous lactate concentration was greater than the arterial; in the third subject, from whom the blood was obtained five minutes after exercise, this condition was reversed.

The work on the relationship between oxygen debt and lactate production was continued by Margaria and Edwards (1934). They demonstrated that there was not a rapid removal of lactate at the beginning of recovery corresponding to the rapid fall in oxygen consumption. Therefore, there was a delay in lactate removal. They also observed a curious fact that the speed of removal of lactate in man is inversely proportional to the amount of lactate formed. This work was repeated later by Newman, Dill, Edwards and Webster (1937). They did the clever experiment of having the subject run to exhaustion in order to saturate the body with lactate and then have the subject exercise at a more moderate rate. The rate of disappearance of lactate under these conditions was faster than during rest, and, in fact, was greater with more severe grades of the exercise in the second period. These authors list the following conditions as possibilities for explaining the removal of lactate: loss in sweat and urine, utilization by heart muscle, utilization by active skeletal muscle for fuel, and the more rapid circulatory rate. Bang (1936) has also described a decrease in lactate concentration in the blood during work. In his cases, the slope of the decrease is approximately the same as during a resting recovery period. If the work is continued long enough, the values at the end of the exercise may be the same as the basal level, demonstrating that a full recovery to the basal level may take place during exercise. However, when the exercise is severe, a secondary rise is obtained during the exercise. These changes in lactate concentration are accompanied by small changes in pyruvate concentration (Johnson and Edwards, 1937) and are not affected by the removal of the sympathetic nervous system in dogs (Edwards, Brouha and Johnson, 1938).

In all of this work, the assumption was made that the lactate in a sample of blood is in equilibrium with the lactate in the tissues. Unfortunately, direct determination of the lactate concentration in the tissues and blood has not always shown this to be true. Margaria and Edwards (1934) studied the rate of disappearance of lactate from the whole body of mice following a period of muscular stimulation. The mice were dropped into a freezing mixture, and the total lactate determined. Since

the rate of disappearance of lactate from the body of the mouse corresponded to the same slope for disappearance of lactate in the blood of man, they concluded that the blood lactate concentration represented body lactate concentration. Sacks and Sacks (1937) approached the problem more directly by comparing the lactate concentration of a cat's stimulated muscle with that of venous blood. They report marked differences between these concentrations: for example, the muscle had 324 mgm. per cent lactate while the blood only had 59 mgm. per cent. Even after two minutes of recovery, there was still a wide difference between the concentrations in muscle and blood. When these experiments were repeated in rabbits, closer correlation was observed than in cats. Newman (1938) studied the lactate concentration in the blood of rats and in the working muscles. He reports good agreement between these two variables up to increments of 80 mgm. per cent. Bang (1936) agrees with Sacks and Sacks that there is an unequal distribution of lactate between blood and tissues.

It is difficult for the reviewer to understand why there should be a marked difference between the lactate in the muscle and in the venous blood. The rate of diffusion of lactate from isolated muscle is a function of the amount present. Eggleton, Eggleton and Hill (1928) suggested that the diffusion takes place through the lymph interspaces, where diffusion is rapid, and through the muscle fibers, where diffusion is 100 times slower. The net diffusion rate depends on these two variables. In a fatigued muscle, the muscle fibers may swell, causing the lymph spaces to decrease, and thus impede diffusion. It may be that these changes are more pronounced in the cat than in mice, rabbits and rats. It may also be that the circulation through the muscle of the cat is less efficient than in the other species. It would be of interest to compare the capillary bed of a stimulated cat's muscle with that of a dog or rat. It is well known that a cat has very little capacity for long continued exercise. Hodes (1939) reports that a cat could run for four minutes on a circular treadmill without noticeable ill effects. A calculation from his data gives the rate at approximately 3 miles per hour. This is in marked contrast to a dog which will go for 17 hours at this rate without noticeable ill effects on the output of work. It is very possible that the circulatory and diffusion factors in the cat may be different from those in the dog and rat. Unfortunately no information is available on this important point for man.

The lactate that appears in the blood is connected with the anaerobic mechanisms in the muscle, a fact which is obvious in very severe grades

of muscular work in which the supply of oxygen does not meet the demand. It is not so obvious for light grades of muscular work. Sacks, Sacks and Shaw (1937) report that at the beginning of stimulation of a muscle, there is always a slight increment in lactate formation which is not increased by continued stimulation. Floek, Ingle and Bollman (1939) have also made a similar finding. Therefore, the beginning of muscular exercise is also an anaerobic condition, since the circulatory and respiratory adjustments require a short period of time before they reach sufficient capacity to carry oxygen to the muscles to meet the new demands. This separation between the aerobic and anaerobic phases in muscular energetics has been reviewed elsewhere (Gemmil, 1939; Sacks, 1941). Since the lactate comes from carbohydrate, carbohydrate is of importance in the early phases of muscular contraction as well as in the prolongation of muscular work.

Excretion of nitrogen in urine. A method which has been used extensively for the determination of the rôle of protein in the metabolic changes during muscular activity has been to measure the nitrogen output in the urine before, during and after exercise. Cathcart (1925), in his review, concluded that the small increment in nitrogen excretion observed during exercise did signify that protein was used as a fuel for muscular work. He discussed the complexity of the problem and suggested that protein might be retained during exercise, inasmuch as the work hypertrophy of muscle would need additional protein. Therefore, the true utilization for muscular work would be masked by the synthetic utilization of the nitrogen-containing residues of protein metabolism.

There are many factors which may change the output of a substance in the urine during and following work, besides an actual increase in the amount of this substance formed by the body. Kidney function may be affected by exercise. Grande and Rehberg (1936) report that light work does not affect kidney function, but heavy work produces signs of renal insufficiency. Hellebrandt, Brogdon and Kelso (1932) described an albuminuria produced by prolonged steady work as well as rapid and exhausting work. These authors advance the idea that the increase in acidity changes the permeability of the renal tissues to proteins. Edwards, Richards and Dill (1931) also have described albuminuria following exercise. If exercise may bring about a change in permeability of the renal tissues to proteins, the output of many other substances may also be affected. Therefore, in evaluating nitrogen output experiments, more significance should be attached to the output for long periods after the exercise than for short periods immediately following exercise.

The fact that exercise increases the output of nitrogen has been confirmed by several observers since Cathcart (1925) wrote his review. His own careful studies with Burnett (1926) demonstrated that exercise increases nitrogen excretion and that there is a parallel increase in sulphur output. Garry (1927) studied the output of nitrogen during static work by allowing the subject to pull against heavy springs and to hold weights suspended by a foot lever. A tremor developed in the contracting muscles which became progressively worse during the time of the experiment. This type of work produced a small rise in the output of total nitrogen, the general average, changing from 15.12 grams per day before the exercise, to 15.77 grams on the day of the exercise, then increasing to 16.36 grams on the first day after exercise, and not falling back to the basal level until three days after the experiment. Wilson (1932), whose subject carried out work on a hand or bicycle ergometer, demonstrated a definite rise in nitrogen output followed by a secondary fall. The work done by his subjects varied from 22,000 to 34,000 kilogram meters per day, and the work periods extended from 4 to 18 days. However, the changes in the excretion of nitrogen and sulfur did not parallel the amount of work done which indicates that there is no direct relationship between these two variables. Later (1934) Wilson reported results of an experiment in which a cyclist rode a bicycle ergometer for 8 hours doing up to 366,850 kilogram meters of work in the day. Again, an increase in the output of nitrogen occurred on the working day followed by a fall below the basal level on the third day after the exercise. The subject carried out the work with diets containing various amounts of proteins. The greatest absolute increase was obtained when the diet was highest in protein. Cuthbertson, McGirr and Munro (1937) have described a nitrogen sparing action of carbohydrate on protein metabolism during exercise. If a meal of carbohydrate is ingested before the work, the rise in nitrogen and sulfur in the urine following work does not occur. These experiments are of special interest, for they indicate a possible explanation for the rise in nitrogen in the urine during and after work. In long continued work, the carbohydrate supply is exhausted, and extra protein is converted into carbohydrate or carbohydrate intermediates to supply energy. This fact would explain the long continued output of nitrogen after exercise. It may also explain the long continued increase in oxygen consumption after work. Herxheimer, Wissing and Wolff (1926) have found an increase of 10 per cent above the basal level even 36 to 48 hours after work. Edwards, Thorndike and Dill (1935) have described a 25 per cent rise above the basal

level 15 hours after activity. A continued oxidative deamination of amino acids would produce these changes in oxygen consumption.

The conclusions to be drawn from this work are that long continued muscular exercise produces an increase in the output of nitrogen in the urine. Since the severity of the work does not affect the output, there is no direct relationship between exercise and nitrogen output. An indirect relationship is postulated; exercise reduces the carbohydrate supply of the body, extra protein is deaminized to supply carbohydrate or carbohydrate intermediates. The evidence for this assumption is based on the fact that ingestion of carbohydrate abolishes the increment of nitrogen output. This idea may also explain the long recovery period observed by some authors.

GENERAL SUMMARY

From the survey of the literature it is obvious that the use of carbohydrate is of primary importance as a fuel for muscular exercise in man. The evidence comes from the slight increase in efficiency on a carbohydrate diet, the prolongation of muscular effort when carbohydrate is ingested, the fall in blood sugar during long continued muscular exercise and the production of lactate at the beginning of exercise and during severe exercise. The evidence that protein is used during exercise indicates that it is of secondary importance, probably to supply carbohydrate or carbohydrate intermediates. The results of experiments on fat utilization during muscular work have demonstrated that this substance is used indirectly. There is no experimental evidence at the present time for the direct utilization of fat by mammalian muscle. However, the indirect utilization of protein or fat must be an efficient process, since the exclusive feeding of these substances to man does not have a marked effect on muscular efficiency during short periods of exercise.

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RECENT ADVANCES IN KNOWLEDGE OF THE LIVER¹

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Rather than attempt a complete review of the extensive recent literature, the author in the present article desires to piece together an account of some of the more important of the newer contributions toward our understanding of the liver's physiology. The pathology will hardly be touched upon and many topics will be referred to merely with brief mention of the literature. Recent reviews not quoted in the text are: Greene (1941), Hawkins (1931), Ivy and Crandall (1941), Josephson (1941), Mann and Bollman (1939).

The rôle played by the liver in the maintenance of blood levels of sugar, urea and protein. From the time of Bernard's discoveries in the 1850's physiologists have investigated the nature of the liver's control of carbohydrate metabolism, but much of the evidence gained was conflicting and confusing. The cause of this is now clear. For one thing it was unknown that various hormones and enzymes, activators and the like were taking part in the liver's activities. The gradual discovery of these and their interactions, the rise of the concepts that the blood-level of a substance of itself releases regulating mechanisms, and of a balanced endocrine action, finally explained many conflicting observations. Another important advance was the gradual perfecting of methods and techniques whereby the physiological integrity of the liverless animal could be maintained for periods long enough to allow one to make a satisfactory series of observations. This was done by F. C. Mann (1921) who finally with associates (1925) established beyond all doubt that the liver is the chief organ concerned with the maintenance of the blood-sugar level; their method was extended and their results confirmed by Soskin and his associates (1927; see review of the literature by Soskin, 1941).

But with all our advances the complete pattern of the mechanism of the extrinsic control of the liver carbohydrate metabolism cannot yet be

¹ Supported in part by a grant from the Rockefeller Foundation Fluid Research Fund.

outlined. For example, from Professor Fiessinger's laboratory in Paris comes a dissertation (Bareillier-Fouché, 1939) reporting what appears to be careful work and convincing evidence that insulin injected into the portal bloodstream of the isolated, perfused cat's liver increases the output of sugar in the hepatic outflow: a veritable hyperglycemia is produced. On the other hand de Bodo and his co-workers, making their observations with the organ *in situ*, show that liver-glycogen mobilization occurs in the absence of adrenal medulla and liver nerves (1938), that in the absence of anterior pituitary liver-glycogen is not readily mobilized by adrenalin (1941a), and that insulin given in doses too small to evoke a more than slight drop in blood sugar in normal dogs, will produce a hypoglycemic shock in completely hypophysectomized dogs. From these observations they conclude that the factors normally effective in mobilizing liver glycogen are ineffective in the absence of the anterior pituitary, hence the sensitivity to insulin observed in hypophysectomized animals (1941b). The findings of de Bodo and his colleagues are supported in part by the independent observations of A. Allen *et al.* (1941) who made their studies upon rats.

Jensen and Grattan (1940) reported evidence that the anti-insulin effect of the anterior pituitary in normal mice may be attributed to the adrenotropic principle of the gland which is mediated through the adrenal cortex. Grattan and Jensen (1940) among others then found the specific principle of the adrenal cortex, which responds to the adrenocorticotrophic substance. They suggested that the anti-insulin effect thus produced is probably due to the ability of these substances to promote formation of liver glycogen. Hartman *et al.* (1940) came to a similar conclusion from studies on normal, fasted mice. Grattan *et al.* (1941) show that the adrenotropic factor of the anterior pituitary fails to produce an anti-insulin effect (and to promote glycogenesis) in adrenalectomized mice, but that under the same condition the administration of corticosterone protects them against insulin hypoglycemia and liver glycogenesis.

Corey and Britton (1941) find that isolated cat livers perfused with Ringer-gum-glucose solution, to which cortico-adrenal extract had been added, show greater increases in glycogen content than when other perfusates are used, the average increase being 60 per cent. On the other hand no glycogenesis could be demonstrated in these cat livers when insulin was added to the perfusate, on the contrary a decrease of glycogen content of about 30 per cent was observed. This latter observation agrees with the findings of Bareillier-Fouché (1939) mentioned above,

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Elements controlling blood coagulation. It is known also that the liver is the only source of fibrinogen (Foster and Whipple, 1922) and of prothrombin. Further corroboration of the fact that the liver elaborates these substances is just now at hand: Brinkhaus and Walker (1941) have shown that the content of liver lymph in prothrombin and fibrinogen is equal to that of blood plasma, while that of femoral lymph is only $\frac{1}{10}$ of that of the blood plasma. Hence the use of the blood level of these substances, as a basis in methods for the estimation of hepatic dysfunction, may be considered well founded.

Heparin was first extracted in Howell's laboratory chiefly from liver, hence its name (McLean, 1916; Howell and Holt, 1918). But recent researches have shown that heparin is produced and stored specifically in the mast-cells, which are scattered generally along the walls of smaller blood vessels and especially along the walls of veins and in the intercapillary spaces. While Glisson's capsule, enclosing the liver lobules, is especially rich in these cells, the lung tissue per unit weight is richer in mast-cells than liver tissue, and therefore is a better source of heparin than whole liver (see Jorpes and Bergström, 1936; Holmgren and Wilander, 1937). We can see now that while small injuries to tissues would tend to the release of the clot-promoting thromboplastic substance and thus to the formation of clots, the presence of the mast-cells in such cases may lead somehow also to release of heparin, thus preventing clots from forming in the capillaries of minute, circumscribed areas. On the other hand large wounds in muscle masses, where mast-cells are scarce, release amounts of thromboplastin in great excess of the heparin liberated and thus clotting in those cases is insured.

It has long been known that, when dogs are thrown into shock by Witte's peptone, the clotting time of the animal's blood is markedly increased. The nature of this phenomenon was cleared up by Wilander (1938) who showed that the heparin content of the blood of peptone-dogs had increased enough to account for the delayed clotting time and that the mast-cells of their livers had emptied themselves of almost all their stores of metachromatic, granular material, which on account of its special affinity for toluidine blue is believed to be heparin itself. (See also Jorpes, 1939.)

Jacques, Charles and Best (1938) finally have shown that the outflow from the hepatic veins of dogs in anaphylactic shock contains liberated heparin, and that, although dogs without livers can also be thrown into anaphylactic shock, their vena caval blood no longer contains heparin and its clotting time is not increased. They believe therefore that the mast-cells of the liver are the source of the heparin found in the non-

hepatectomized peptone-dogs. Why there is this specific action between Witte's peptone and the mast-cells only of the liver is a problem awaiting solution. (For confirmative evidence see Jacques and Waters, 1941.)

Calder and Kerby (1940) studying patients suffering from certain diseases which develop hemorrhagic syndromes found that intravenous injections of comparatively small amounts of nicotinic acid will remove the hemorrhagic symptoms as well as a number of other pathological conditions. In an attempt to solve the nature of this action of nicotinic acid, by working with samples of the blood of these patients in test tubes, they found that, unlike in the human body, it took enormous doses to shorten the clotting time to a normal period. The authors inferred from this that the rôle of nicotinic acid is probably a pharmacodynamic one, in which minute doses of the drug act first upon the liver causing it to produce and liberate relatively large quantities of prothrombin, or much less antithrombin, and thus to enable the shed blood to clot. This hypothesis should be tested by experiments on the isolated surviving liver.

Vitamins. Nicotinic acid is a substance grouped with the water-soluble B complex of vitamins, and sometimes referred to as vitamin-pp. We come thus to a consideration of the rôle played by the liver in the storage, distribution and activity of vitamins in general.

About sixty-four years ago Boll observed that the pigment in the retina, visual purple, was photosensitive. A year later Kühne discovered that this pigment could be dissolved out from the retina by a solution of bile salts. Following this discovery the power of the eye to adapt itself to vision under dim illumination was ascribed to some hidden chemical reaction of this photosensitive substance. But the first insight into the substances involved in this chemical reaction has been revealed only during recent years.

Beginning with the work of Fredericia and Holm (1925) and culminating in that of Wald and his associates, it has been shown that visual purple is generated in the dark by a synthesis of vitamin A and a carotenoid substance called "retinene," and that the bleaching of the pigment upon exposure to the light is due to a breaking down of this vitamin A-carotenoid combination.² If now the quantity of vitamin A in the body begins to fail, the resynthesis of visual purple also begins to fail and thus fails also the power to see in dim light (see Wald, 1935).

² There may be doubt as to the exact chemical nature of this dependence. Krause and Sidwell (1938) could not demonstrate that vitamin A is formed by the photochemical reaction of visual purple or visual yellow. That some fundamental relation obtains however is beyond all question.

In 1932 Drummond and others were able to show that the absorption of fat-soluble carotene by the gut could only be carried out if bile acids were present. These authors assumed that the bile acid formed a water-soluble, diffusible compound with the carotene, the absorption and transport of which into the bloodstream was thereby facilitated. Later Drummond *et al.* (1934, 1935) showed that the distribution of fat and vitamin A in the three lobes of the rat's liver is reasonably uniform; that carotene in certain form injected into the portal circulation is rapidly taken up by the Kupfer cells, and when thus taken up tends to disappear within the next few days. No correlation with vitamin A was found at that time but other experiments showed that the removal of one lobe of the liver of itself brings about a loss of vitamin A in the remaining lobes.

Young and Wald (1940) now find that removal of one lobe of the rabbit liver reduces vitamin-A 25 per cent and carotene 50 per cent in the remainder of the organ, while blood-vitamin-A rises 3 to 7.5 times its former values. These authors further find that electrical stimulation of the splanchnic nerves increases blood vitamin-A, while electrical stimulation of the cervical sympathetics has no effect. Schneider and Widman (1934) demonstrated that thyrotropic hormone plays an important part in the cleavage of carotene into vitamin-A and finally causes a discharge of vitamin-A from the liver. Young and Wald conclude that the liver is not only a store-house for vitamin-A but also that its mobilization is affected in ways similar to those which mobilize sugar in the liver.

From these experiments it appears that bile acid, in its rôle as an agent promoting fat resorption, as one would expect, also promotes the resorption of fat-soluble substances. Thus, defects in the liver, leading to a paucity of bile acids in the gut, will lead to a deficiency of carotene and vitamin-A in the blood, which in turn leads to further depletion of stores of these essential substances in the liver and finally to a reduction of their blood-levels.

One recalls that Moll *et al.* (1933) produced a concentrate from fish liver oils very high in vitamin-A, which they called "Vogan." Domagk *et al.* (1933) observed that the Kupfer cells of the liver show a selectivity for fat and that these cells seem primarily concerned with vitamin-A storage and destruction. Vitamin-A given with fats is more easily absorbed and assimilated. But when vitamin-A is given in overdoses *a*, according to Domagk (1933) lipemia follows, and *b*, according to Lasch (1934) serum-cholesterin increases, both due mainly to liver action.

Since the chief natural source of the fat-soluble, antirachitic vitamin-D is in the oils of fish livers the question pertinent in the present inquiry is, why is this vitamin not stored also in the livers of mammals. The answer is that the lung tissue of mammals contains an enzyme which destroys vitamin-D; this depletes the blood and finally the liver and other tissues of lung-breathers of their stores and prevents accumulation of vitamin-D. The gill-breathing fishes on the other hand have no such enzyme; their liver fats therefore are able to accumulate stores of this fat-soluble vitamin. The vitamin-D series is composed of definite sterins which are fats and thus will be taken up by other fats in greater quantity, especially in such livers as store fats instead of carbohydrates. A consideration of the inactive provitamin of vitamin-D and the effect of irradiation upon it, and of the source of the active vitamin in cod livers (they themselves contain mere traces of the provitamin) would take us too far a-field at this time.

Perosis, known otherwise as "slipped tendon" or "hock disease" appearing in chicks, is reported by Hogan *et al.* (1940) to be due to the lack of a factor, which they call B_p , in addition to the lack of manganese in the diet. According to Stepp *et al.* (1938), water-soluble B_1 , vitamin- B_2 -complex and B_6 are stored up or elaborated, or both, by the liver more than by any other organ. From the experiments of György and Goldblatt (1939) it is known that rats fed on basic rations lacking the "rat growth factor," B_w , develop pathological changes in their livers, which latter can be prevented by adding yeast or yeast extract to the diet. The "chick antidermatitis factor," the "rat growth factor" and "pantothenic acid" probably all belong to one group of substances somehow closely related.

The chromatrichia factor for rat and the growth-promoting factor for chick derived from yeast are identical, if one accepts the work of Ansbacher (1941) and others, and is a vitamin of definitely known composition, namely, p-aminobenzoic acid. This aromatic compound is greatly facilitated in its resorption by the intestine in the presence of bile salts.

Recently Rich and Hamilton (1940) have been able to produce experimentally a form of Laenec's cirrhosis of the liver in normal rabbits by feeding a diet in every respect apparently quite adequate and including the isolated yeast-factors, B_1 , B_2 , and B_6 or nicotinic acid. But if *full yeast* is added to the diet, the animals no longer develop the cirrhosis. It was therefore concluded that yeast contains as yet some undetermined factor which plays the rôle of an anticirrhotic agent. Maschella and Macguire (1941) could not confirm this. But György and Goldblatt

(1941) report increased incidence of necrotic and cirrhotic lesions in the livers of rats when the normal diet, containing 18 per cent casein, was reduced to one containing 10 per cent and in which lard was substituted for butter. Cystin added to this latter diet increases the incidence; choline added to it, decreases the incidence of the liver injuries. These observations agree with the extensive work of Blumberg and McCollum (1941) who with high fat, low protein diets produced cirrhosis in more than 30 rats and have prevented cirrhosis in a like number of animals by the addition of choline.

Similarly Miller *et al.* (1941) find that rats on a given carcinogenic (butter fat) diet within four months show a 90 to 100 per cent incidence of hepatic tumors which can be reduced by simply increasing the casein or other protein content of the diet. Addition of whole dried brewers' yeast to the carcinogenic diet offered only partial protection. They conclude that the protective elements in such diets are high content of both protein and vitamin-B complex.

From numerous recent public lectures, published reports and reviews (by Dam, 1940; Almquist, 1941; Doisy *et al.*, 1941) it is now well known that several forms of hemorrhagic diatheses are due primarily to lack of a special substance called vitamin-K. The natural vitamin-K is a fat-soluble compound and therefore requires bile acid for its absorption. But Doisy *et al.* have synthesized a quinoid compound in which naphthaquinone is joined to phthiocol. This compound has all the essential characteristics of vitamin-K, but is water-soluble. The point of interest for us at the moment is that K-avitaminosis patients bleed because a clotting factor is absent in their blood. Whipple (1912) had shown that infants suffering from *icterus gravis* had blood of long clotting time. Only in recent years however has it been found that in these cases the immediate trouble is lack of prothrombin. Later studies showed that Vitamin-K normally stimulates the liver to liberate prothrombin into the blood-stream, whereupon the bleeding promptly ceases.

But a third important factor had to be revealed for the final solution of the etiology of these hemorrhagic diseases. If the bile is absent or deficient in the intestine, as in gall-duct obstruction, sufficient vitamin-K is not absorbed. And if, as in the case of the new-born infant, where the diet lacks the vitamin, and the normal intestinal flora do not develop soon enough to provide it, no vitamin-K is at all available, hence the lack of prothrombin in the blood. This explains how, in both these categories of cases, hemorrhagic symptoms develop. By introducing vitamin-K directly into the blood, or better by intramuscular injection,

one has an effective procedure which makes the patient safe from bleedings occasioned by trauma or surgical intervention until he can normally supply sufficient quantities of the vitamin from his own alimentation. The mechanism whereby vitamin-K is able to stimulate the liver to produce prothrombin is still to be solved.

Miscellaneous. Depancreatized animals treated with insulin under certain conditions commonly develop marked hypolipemia and fatty infiltration of the liver. To test out the idea that this change in fat metabolism may be attributed to lipocae deficiency (Dragstedt *et al.*, 1936), or to defective digestion and absorption of fats (due to absence of pancreatic juice in the intestine) J. G. Allen *et al.* (1941), having made complete external deviations of the pancreatic juice in dogs, found in these cases that the blood and liver lipoids nevertheless remain within the normal range. It would appear here then that the bile even in the absence of pancreatic juice still plays an important rôle in fat absorption and thus in the maintenance of lipemic levels. The problem of fatty infiltration of the liver thus is not fully solved. (But see addendum.)

The liver is known for its detoxifying and inactivating powers of certain substances coming to it in the blood stream. The following serves as a recent example. Natural estrogens given by mouth are ineffective. The elegant proof as to the cause of this inactivation is as follows. If ovaries are transplanted intra-mesenterially so that their venous drainage enters the portal system they cease to have estrogenic effects, from which one infers the liver in some way renders the hormone inactive. M. J. Allen (1941) now brings forth evidence proving that the synthetic product, stilbestrol, which in every way has the same effects as natural estrogen is not inactivated while passing through the liver, and therefore may be given either per os or implanted as pellets intra-mesenterially or subcutaneously and still have its full effect. Just what happens to the natural estrogen while passing through the liver is another problem in liver physiology. (See also Selye, 1941; B. Zondek, *et al.*, 1941.)

This incomplete account of the multiple known functions of the liver may not be concluded without at least brief mention of what may be regarded as one of the most important of all, the hematopoietic function. Since the fundamental discoveries of Whipple and his associates and those of Minot, Murphy and Castle, investigations have been continued by numerous workers to unravel the mystery of the antipernicious anemia principle which is agreed on all sides to be elaborated, stored and

distributed by the liver. Jacobson and Subbarow (1941) discuss the possible chemical nature of this principle, judged from all knowledge gained thus far, and Formijne (1940) contributes experimental data on the nature of "the extrinsic factor" and "the reaction of Castle."

The work briefly reported by Crandall *et al.* (1941) doubtless will be of great importance 1, in rendering an experimental animal available (and which may be substituted for human patients) for the assay of liver extract for therapeutic use, and 2, by experimentation in clarifying "the etiology of the macrocytic anemias of man."

The vasculature and circulation of the liver. Earlier observations led to an estimate that about one-third of the blood of the liver was delivered by its artery. However from the observation of Grindlay *et al.* (1941) on the unanesthetized dog, the present author estimates that the ratio of hepatic arterial flow to portal vein flow may be more nearly one-seventh than one-third!

The oxygen tension in hepatic arterial blood is ca. 85 per cent; that in portal vein blood, ca. 50 per cent. The final outlet from the liver is the large hepatic vein, from two-thirds to six-sevenths of whose blood thus for the most part has passed through two capillary beds. The blood vessel walls are well supplied with nerve fibers. But the effective distribution of these nerves to the various vascular systems is in considerable doubt as to quantity and kind. Besides this the liver is richly supplied with lymphatics.

The veins of the liver of many vertebrates have a peculiar distribution of smooth muscle. In the hepatic veins and venules of the dog and amphibious mammals, the smooth muscle cells are not arranged in continuous sheets as in ordinary veins but grouped into slender spirally arranged bands and, especially where the hepatic venules empty into the hepatic vein, the band becomes much thickened and quite annular in disposition around the lumen, so as to provide a sphincter. Authors do not yet quite agree as to the structure of the vascular walls in the human liver but this much is certain, namely, that while the hepatic vein has much smooth muscle in circular and longitudinal sheets, the annular sphincters are also present at the vena caval ostia. These sphincters when closed, and at times they doubtless do close up the lumen completely, thus act not as the one-way membranous valves of ordinary veins, but as canal-locks or sluice-boxes which when closed reduce the rate of transport of fluid; they act thus in somewhat the same

way as the sphincters of the alimentary canal. (For discussion and literature on these structures see Tischendorf, 1939, and Pfuhl, 1932.)³

Recent studies have corroborated and extended this knowledge of the special sphincter and sluice-mechanisms. Block (1940) describes sphincter-like activity of the walls of frog's liver vessels evoked by the action of adrenalin and acetyl beta-methyl choline. Deysach (1941) working with livers of cats and rabbits, describes small endothelial tubes which are situated in the walls of the sublobular and central veins. The lumina of the latter communicate with those of the former by means of ostia which are guarded by sphincters. These ostia when dilated may permit the small endothelial tubes under certain conditions to be filled with blood. Upon closure of the sphincters large volumes of blood may be sequestered from the regular circulatory pathways of the liver and thus from the general circulation. Under other conditions the ostia may dilate again and the side chambers empty themselves, thus suddenly increasing rate of outflow from the hepatic vein. What the ratio of volume at maximum capacity of these chambers may be to that in the other hepatic venous pathways is difficult to determine but no doubt a method can be devised for an approximate estimation. In a histological study of turtle's liver Tyler (1941) showed that the distribution of smooth-muscle in the walls of portal and hepatic veins was much like that in the dog's liver, and the structures and arrangements of biliary ducts, arteries, parenchyma cells and lobules much like those in the human liver. The walls of the portal venules are practically devoid of all smooth muscle and the hepatic venules and capillary sinusoids have their smooth muscle cells arranged entirely in sphincter and spiral bands. The sphincters at the mouths of hepatic venules are thick, annular bands. (See plate 1 in Tyler, *loc cit.*) If the sphincters found in mammalian liver help us to explain the volume changes of the organ evoked by various agents (Bauer *et al.*, 1932; Deysach, 1941) these structures found in the turtle's liver should explain equally well, the volume changes exhibited by that organ under various conditions (Snyder, 1938).

Intrinsic vascular responses; their effects upon rates of metabolic exchanges. The presence of smooth muscle and the reactions evoked by autonomic drugs indicate an autonomic nervous supply to the sphincters. Anatomists have shown such a nervous supply to the

³ L. B. Arey in *Anat. Rec.*, 1941, **81**: 21, summarizes and discusses the intrahepatic musculature among mammals and illustrates the "throttling mechanism" with reproductions from wax reconstructions of them.

vasculature of the mammalian liver, although the finer details of the nerve-endings are still lacking (Pfuhl, 1932).

The fact that the liver responds to cholinergic agents suggested on the other hand that the hepatic outflow during vagal stimulation of the liver should contain "vagus-stuff." Experiments carried out on the turtle's liver proved this to be the case (Snyder, 1936). Since adrenergic agents when injected into the portal vein also evoke volume changes and flow rate differences it may be inferred likewise that during sympathetic nerve stimulation the hepatic outflow would contain a sympathin-like substance. Experiments by Cannon and Rosenblueth have shown this to be the case. (For literature see Rosenblueth, 1937.)

From the known general effects of these autonomic agents upon other organs and tissues, one would expect them to have antagonistic effects, but the conflicting reports in the literature have abundantly shown however that this is not easily demonstrated for the activities of the liver. The explanation for this conflict in the evidence is no doubt to be found in the fact that many of the experiments have been carried out with the organ *in situ* where it is under the influences of the manifold and variable activities of its fellow viscera, to which it is more than any organ highly sensitive. (See Bauer *et al.*, 1932; Katz and Rodbard, 1937; Grindlay *et al.*, 1941.)

Observations on the liver *in situ* are essential and important but, for an answer to the exact effects of autonomic agents or of autonomic nerve stimulation, especially upon the quantitative uptake or output of substances by the liver, experiments on the isolated surviving organ are more hopeful of reproducible results.

There is one source of error in both preparations, however, that has not received enough attention. In evaluating flow-rates and volume changes and probably also fate of substances, the exudates and lymph flow of the organ too often have not been observed and analyzed. Differences of observations among various works probably would largely disappear if changes in lymph and exudate flows were observed simultaneously with volume or flow-rate changes.

Figure 1, heretofore unpublished, is a reproduction of a smoked drum record, made during an experiment (2/1/1940). A continuously recording flow-meter was put in the inflow path; an automatic tilting vessel records rate of outflow. The upper trace line is a continuous record of the pH of the hepatic vein outflow; the second trace line records the lymph outflow in drops from a drainboard upon which the organ rests, the cut end of the lymph-vessels being open; the third trace-line records

the rate of outflow from the hepatic vein, each down stroke on the trace-line indicates the discharge of the tilting vessel of about 9 ml. capacity; the fourth trace-line records the time in ten-second intervals, and the fifth trace-line is a continuous record of the rate of inflow recorded by what is called a "rotameter." All the recording instruments have been carefully calibrated.

The changes in pH during the experiment vary from a high of 7.45 to a low of 7.39. The inflow recorder shows many spontaneous rhythmic oscillations, which represent varying resistances in the hepatic venous bed. At the arrow 30 gammas of acetyl beta-methyl choline

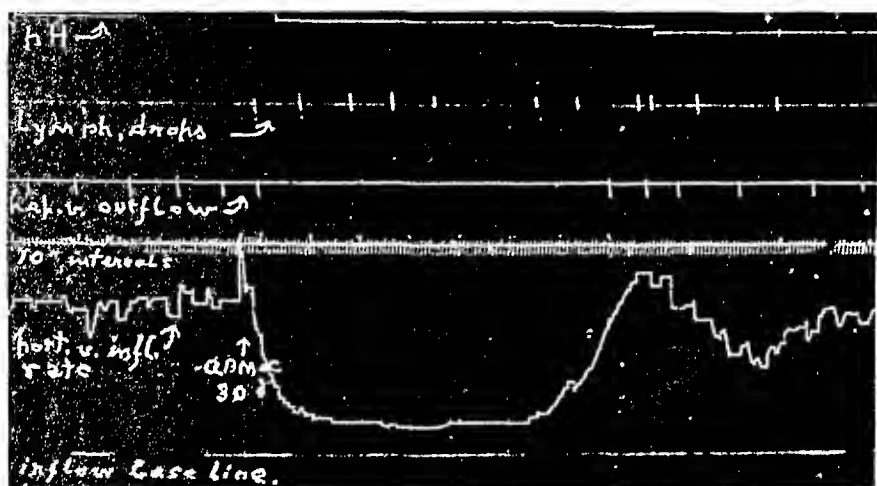


Fig. 1. Shows drum record of effect of 30 gammas of acetyl-Beta methyl choline on rate of inflow to and outflow from liver. See text for detailed description. Read left to right.

(ABMC) were injected into the portal vein. The effect of the agent comes on suddenly, causing at first a brief increase rate of inflow (dilatation of capillary sinusoids?) and at the same time a momentary increase in rate of outflow. This short period of increased outflow would be the result if the sphincters of the hepatic venules began to constrict in a peristaltic wave moving from their smaller branches out toward the hepatic vein itself. Following this, the rates of both inflow and outflow decrease abruptly and profoundly; there is increase in the lymph outflow lasting until the rate of hepatic vein outflow begins to increase. Careful inspection of the records gives one the impression that the blockage to flow of fluid is in the hepatic venous system rather than in the portal

venous system. The head of inflow pressure having remained the same, the observed increase in transudate and lymph flow would be expected. The whole picture seems to indicate that the cholinergic drug, and therefore also motor vagal nerve impulses, stimulate the hepatic venous sphincters to constrict; this action erects barriers to the flow of fluid. The absence of smooth muscle in the turtle's portal venules and portal vein allows of no constriction or activity in those vessels; we assume they remain passive.

Figure 2 is a reproduction of a heretofore unpublished drum record showing the liver's response to 33 gammas of ABMC while in an oncom-

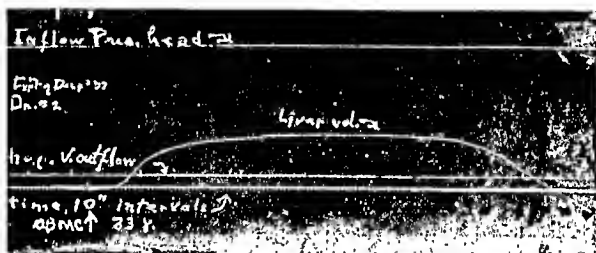


Fig. 2. Oncometer drum record, showing effect of cholinergic agent on liver volume; upward movement = decrease of volume. Time in 10 second intervals; drum greatly slowed down toward end of record. Signal = 33 gammas of acetyl-Beta methyl choline injected into portal vein. Third trace from bottom at left = tilting vessel recording hepatic vein outflow. Top line shows constant head of pressure at inflow. Read from left to right.

eter (Snyder, 1938). The outflow from the hepatic vein is recorded by tilting vessel. The upward movement of the plethysmograph recorder indicates decrease of liver volume. Exudate and lymph outflow, if any, entered the oncometer chamber and thus would have the same effect as increasing liver volume. The even course of the volume recorder before injecting the drug indicates that exudate was very small indeed and if it increased, during the drug effect, it prevented the recorder from exhibiting the full decrease of liver volume that the drug evoked.

The volume during the effect of epinephrin in similar experiments was apt to diminish, a prevailing increase in volume could often be

stopped by injection of this drug (Synder, 1938). "Epinephrin reversal" effects on flow through liver, when minimal effective doses were used, were observed by Snyder and Martin (1922). In these earlier experiments turtle livers were isolated and perfused at constant head of pressure (ca 12 cm. water column) from a set of reservoirs containing perfusing fluids of high and low pH levels and also with and without epinephrin. Concentration of the agent set at ca $1 \cdot 10^{-8}$ to 1 per-fusate, and pH of ca 7.8 caused a decrease in rate of hepatic-vein outflow; but an increase of hepatic vein outflow, if the pH was ca 7.1.

If there are differences in synchronous inflow and outflow rates and especially if there are periods of profound changes in mean flow rate through an organ, the actual uptake and output of substances in the bloodstream by the organ cells must also vary greatly from time to time. To determine this proposition series of experiments such as described above were carried out, during which at intervals of 10 to 15 minutes samples of the inflow and outflow fluids were taken, analyzed and the data reduced to the standard terms of gram of substance gained or lost in the outflow per minute per 100 grams liver.

The substances thus determined were sugar, lactic acid, chloride, potassium, oxygen and carbon dioxide. The results were recalculated also in the same terms but without taking into account the synchronous differences between rates of inflow and outflow through the organ, that is on the old basis of differences in concentrations between inflow and outflow alone and on the assumption that the inflow rate always equalled the outflow rate. The results obtained by the two methods of calculation vary sometimes enormously as is shown in table 1, by Snyder and Tyler (1940b). Here the gains and losses in the liver output are compared with the input 1, in the case inflow and outflow rates are taken as equal, and 2, in the case that the actual differences between inflow and outflow rates are taken into the account. By the first method the glucose and lactic acid have gained in the output over the intake. By the second method both glucose and lactic acid at times are less, at times more. Put in terms of percent, the difference in values obtained by the two methods varies from 4 to 2800 per cent for glucose, from 5 to 228 per cent for lactic acid.

In view of their findings as to the fate of radio-activated lactate administered in rats, Conant *et al.* (1940) have suggested that it may be that lactate, in its apparent conversion to glycogen by the liver, may undergo a decarboxylation. A final answer to this suggestion may well be had by repeating the experiments on the isolated perfused organ under the conditions here recommended. If the decarboxylation

takes place in the liver, equivalent radioactivated CO_2 ought to appear in the outflow from the hepatic vein.

Following the method thus outlined, comparing actual input to output per unit time and unit weight of liver at room temperature, Snyder and Tyler (1940 a, b) have come to the following conclusions:

Oxygen consumption is at maximum when rate of perfusion of a Tyrode solution, saturated with oxygen, is at ca 20 ml. per minute per 100 grams wet weight of liver. Under these conditions ca 0.23 ml. oxygen is used up per minute per 100 grams liver. This oxygen consumption is greater than that of turtle-liver slices surviving in the Warburg apparatus, namely, ca 0.15 ml. per minute per 100 grams liver. The findings on perfused dog liver (Blalock and Masoo, 1936), after being extrapolated for temperature differences, give a mean as great as 0.8 ml. per minute per 100 grams liver; for rat-liver slices the mean extrapolated figure, however, according to Minami's data is as low as for turtle liver, namely, 0.13 ml. per minute per 100 grams liver.

The rate of carbon dioxide production varies enormously on the other hand, the amount in the outflow sometimes being less than in the inflow. This suggests that carbon dioxide is being absorbed to form, possibly, ammonium-carbamate. The R.Q.s accordingly vary so greatly that they are meaningless. (See Addendum, last paragraph.)

There is nearly always an excess of glucose in the outflow over the inflow to the liver. If the pH of the perfusion fluid is high this excess of glucose output is greatly reduced and may become zero. If the content of the inflow in dextrose is high, and the perfusion rate high, then (according to Parnas and Baer, 1912) the glucose is absorbed by the liver and converted to glycogen. No glycogen determinations however were made. Lactic acid in the output by the liver exceeds the input whether lactic acid is present in the input up to 0.1 per cent or not. The absence of dextrose in the inflow increases the output of lactic acid from the liver. High pH of the inflowing perfusate reduced the excess of lactic acid in the outflow, so that at times there may be a deficit instead of an excess. Adrenergic agents here also markedly increase, and cholinergic agents decrease, the excess output of glucose and lactic acid from the liver over the input. The increase in glucose output, due to adrenalin, exceeds the increase in the mere perfusion rate. This suggests and supports the present generally accepted doctrine that adrenalin (and most probably sympathetic nerve stimulation) causes the liver cells to give up their carbohydrate stores directly. The decrease of glucose and lactic acid output produced by acetylcholine on the other hand so nearly parallels the decrease produced in perfusion rate

that we cannot be sure that acetylcholine (and by inference vagus nerve stimulation) directly inhibits the liver cells in their glycogenolysis. When the output of glucose from the liver undergoes a diminution it is a question as to whether its disappearance is due to a splitting into lactic acid on its way through the organ or to a conversion into glycogen. Our simultaneous determinations of both glucose and lactic acid gives evidence that the decreased output of glucose by the action of acetylcholine cannot be due to glucose splitting into lactic acid, for the lactic acid output at the same time is also decreased.

In order to detect if possible more precisely the *loci* of the effects of autonomic agents when injected in single, small doses into the turtle's portal vein, a series of experiments were carried out in which continuously recording flow-meters were introduced into *both* portal and hepatic veins. The observations and results permit the following interpretation (Snyder, 1941):

Since the portal vessels are devoid of smooth muscle they cannot be expected to respond to this class of agents. The observed responses to cholinergic agents, namely, shortlasting great increases in hepatic vein outflow accompanied by lesser- and longer-lasting decrease of inflow-rate must be due therefore to constriction of the hepatic vein and venules starting in a peristaltic wave at their sublobular origins and proceeding toward the caval orifice of the hepatic vein. This would account for the initial increase in outflow and also for a longer lasting resistance set up which in turn brings about the longer lasting decrease in inflow rate, and also the final effect on outflow, namely, almost complete obstruction, until the agent is destroyed.

The adrenergic effects observed are a slow persisting rise of outflow-rate and slow, persisting fall of inflow rate. To explain this it seems simplest to ascribe the increase in outflow to a sudden release of fluid from the parenchyma cells (which is accepted as one of the effects of adrenalin on the liver). This sudden increase in volume of fluid in the hepatic venules sets up a resistance to the inflow from the portal side, which lasts as long as the increase in the outflow rate. For the evidence and further discussion the reader should consult the original paper.

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ADDENDUM

Positive evidence of the efficiency of lipocaine in controlling fat-deposition in the liver will be found by Isabolinskaya, *Bul. biol. et med. exper. U. R. S. S.* 9: 107, 1940, who finds the pancreatic extract to have the power "in most cases" of inhibiting the development of toxic fat-infiltration of liver. Gavin and McHenry, *J. Biol. Chem.*, 141: 619, 1941, now show that lipocaine has a differential effect on deposition of various fatty substances in the liver, preventing only that produced (in rats) when biotin is fed along with thiamine and certain other substances, whereas the "thiamine type" of fatty livers is prevented with choline and not with lipocaine.

Evans and Slotin, *J. Biol. Chem.*, 141: 439, 1941, in an elegant series of experiments, where they used radioactive C^{14} as a tracer, demonstrate that CO_2 is utilized by minced pigeon liver in the formation of alpha-ketoglutaric acid from pyruvic acid.

THE PRESENT STATUS OF THE SHOCK PROBLEM

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REVIEWS. The subject has been systematically reviewed in a number of texts and monographs (Blalock, 29; Cannon, 42; Harkins, 117; Y. Henderson, 121; Moon, 176; Rehn, 205; Scudder, 226; Turck, 246; Wiggers, 259); it has also been treated in symposia, editorials and numerous papers, more limited in scope (24, 33, 36, 43, 64, 73, 90, 98, 102, 109, 116, 120, 156, 164, 169, 170, 174, 175, 186, 190, 195, 200, 206, 210, 215, 245, 250, 265, 266). In view of these excellent surveys and sources of reference, another comprehensive, systematic, or historical review is neither needed nor intended. A review of existing reviews does leave the impression, however, that, if they have any shortcomings, these are a, that many writers have marshalled and oriented experimental discoveries so that they seem to support only one favored theory of shock; b, that, in the zeal to crystallize conceptions concerning the mechanism of shock, *conclusions* have been accepted too generously in place of *demonstrated facts*, and c, that the experimental conditions under which incontrovertible results have been obtained were not always carefully scrutinized and evaluated. Consequently, another survey of the shock problem will probably be of greatest use if it stresses some of these features. It is the author's intention, after a brief historical survey and orientation, to analyze the experimental conditions under which shock is best studied, to examine the validity of generally accepted conclusions in the light of present day knowledge, to reweigh the experimental support for disputed hypotheses, and, in a few instances, to reassign priority for ideas in accordance with experimental evidence therefor. Such an essay cannot favor any hypothesis; it will rather tend to emphasize discrepancies and gaps of information. Nor can it be expected to bridge these gaps by speculations or citation of opinions.

THE MARCH OF PROGRESS. The author's first review of the shock problem consisted of an essay written while he was a medical student in Ann Arbor. The report recalls the status of the problem in 1903. It emphasized 1, that most of the early hypotheses were originated in an

arm chair or at the desk, and that careful clinical observation and reflective thinking had failed to solve the problem; 2, that a change in thinking was in progress which transferred the fault from failure of the heart to failure of the peripheral circulation, and 3, that physiologists had apparently not yet become interested in a definitely functional disorder. At that time, indices of physiological text-books either did not list the word or, as in Schafer's well-known volumes, it read, "Shock—sec Spinal Shock." The term *shock* had entirely different connotations for clinicians and physiologists.

The early hypotheses, ably reviewed by H. Fischer in 1870 (82) and by Groeningen in 1885 (110) seriously attempted to apply existing physiological knowledge to disease. This had many pitfalls and certainly did not prove adequate. In some instances, as in the frequently quoted papers of Maleolm (1893-1907) (160), it merely resulted in a curious confusion in applying known hemodynamic principles to the circulation.¹ When the hypotheses formulated by them agreed with those later supported by experimental investigation, this was purely fortuitous.

The experimental era began with the extensive investigations of Crile, published in his first monograph in 1890 (55). While his hypothesis resembled that propounded by Groeningen, it did have a background of experiments, many of which have become generally accepted. The experiments of Howell (136) constituted the next major attempt to elucidate the subject in the laboratory, and to this end he employed many procedures still used today. He concluded that shock may be either

¹ His earlier hemodynamic conclusions read, "Any treatment and condition tending to dilate the systemic vessels by diverting the flow of blood from the internal to the superficial parts must tend to increase the blood pressure in the carotid, by causing its contraction . . ." (1905).

Later, in attacking Crile's hypothesis which involved arteriolar dilatation the issue was confused with passive changes in larger arteries. "When a series of vessels in the normal human body is contracted the blood pressure in the affected area is lowered, whilst the flow becomes more free and the pressure rises in some other part or parts of the vascular system." . . . "Again when small arteries contract the volume and pressure of blood within them are reduced and the lumen may even be occluded so that blood pressure is completely removed" (1907). . . . "Thus, in the large and small vessels and practically in all conditions a lowering of blood pressure in any particular artery or vascular area is accompanied by contraction and not a relaxation of the vessels concerned" (1907). Again, "In conclusion I would urge that as the state of shock develops, there arises an intense and increasing stimulus to contraction of the vascular system, which in advanced cases may give rise to a low blood pressure from overaction of the heart" (1910) (1).

of cardiac or vascular origin. He also made the significant observation "that operations which were sufficient to produce shock in one animal might in another have little or no effect of this kind." The efforts to devise a standard procedure that unfailingly produces shock seems as hopeless today as in 1903.

Once an interest had been created, physiologists were not slow to test various hypotheses. The experiments of Porter (1907-08) (202), of Seelig and Lyon (1910) (225), made untenable the concept that exhaustion of the vasomotor center initiates shock. Y. Henderson (1908) (122) stressed decrease in venous return and cardiac output as initial factors and developed his acapnia theory. Meltzer (1908) (172), with an experimental background of observations on intestinal motility, suggested a broader conception of shock which stressed dominance of the inhibitory side of all reflex actions.

During the years of the First World War (1914-18), the curve of interest among physiologists rose sharply with the result that they were not only actively at work in various laboratories in the United States and England but that teams composed of physiologists and surgeons co-operated in the war zones of France to utilize the extraordinary opportunities for studying shock in man. (For reviews see Cannon, 42; Harkins, 117.) These observations produced new problems for reinterpretation in the calm of the experimental laboratory. Such shuttling of problems between the field and laboratories of basic science proved of immense value, and when the smoke of battle had cleared away the following definite advances could be recorded: 1. It seemed highly improbable that shock was due either to *a*, exhaustion of the vasomotor center; *b*, to adrenal-medullary deficiency; *c*, to acidosis; *d*, to acapnia, or *e*, to fat embolism. 2. It seemed quite probable that, in various catastrophies, a toxic factor (histamine) combined with loss of plasma or blood caused a reduction in circulatory volume which impaired the filling of the heart and its output, and that this was the cause of the progressive lowering of arterial pressure and eventual death through asphyxia. (For reviews of that period see Cannon, 42, and Wiggers, 259.) Moreover, following a suggestion of Gesell (96), investigators endeavored to distinguish between initiating and sustaining factors, and many of them began to realize that the initiating agent might not prove the sole cause of death, if indeed it be involved at all.

As far as physiologists were concerned, the years 1923 to 1941 were noteworthy chiefly in an awakening of interest in the possible relation of the adrenal cortex to shock and in the efficacy of cortical preparations in

its treatment (237, 238, 239, 240, 241, 229, 230). The probability that bistamine represents the guilty toxic agent seemed to receive less and less experimental support. The possibility that potassium may represent a common toxic depressant agent was extensively investigated by Zwemer and Seudder (226, 269). Aside from occasional contributions, chiefly of a confirmatory nature, the mechanisms of shock received scant attention in physiological laboratories. On the other hand, the era was characterized by an acute awakening of interest among experimental surgeons, among whom the teams led by Blalock, L. Dragstedt, Freeman, Lenhart, O'Shaughnessy, Phemister, Slome, etc., may be mentioned. The subject was also investigated systematically from the viewpoint of the pathologist through the leadership of Moon. The ways in which these several lines of approach modified our concepts of shock can be gathered best from a perusal of the recent monographs by Blalock, Moon and Seudder. Finally, and most important, it represented a period in which the knowledge gained was applied in a practical way by surgeons. However, as Harkins (117) states in an admirable recent review, "the question of shock is not yet settled, and, while transfusion therapy is helpful, it is not the entire solution of the problem."

DEFINITION:² As Meek has recently said, "every worker has given a definition of shock to illustrate his own conception of the condition, and this is, of course, what definitions are good for." In this spirit, a definition is again suggested which expresses the extent to which we ought to commit ourselves on the basis of a critical examination of available clinical and experimental evidence. *Shock is a syndrome resulting from depression of many functions, but in which reduction of the effective circulating volume and blood pressure are of basic importance, and in which impairment of the circulation steadily progresses until it eventuates in a state of irreversible circulatory failure.* Such a definition recognizes the involvement of systems other than the circulatory and acknowledges the detrimental effects that such derangements have on the organism as a whole. It also stipulates that the neuromuscular, glandular and visceral phenomena may not all be secondary to circulatory changes. At the same time it concedes that progressive failure of the circulation is of basic importance from etiologic, prognostic, and therapeutic standpoints. Following precedent, we shall limit our discussions of shock to its circulatory aspects.

EXPERIMENTAL SHOCK. The conditions under which experiments are performed frequently determine the results and their applicability to a

² For a concise list of definitions see Harkins (117).

broad problem. This is certainly true in the study of shock and allied conditions, and may be one of the reasons for the differing results that continue to be reported. Under the circumstances, the author will be permitted—and perhaps expected—to discuss certain problems of experimentation, concerning which he has formed strong convictions during more than 30 years of active participation in research on the circulation.

The problem of anesthesia. It is frequently claimed that the reactions of shock can only be studied in unanesthetized animals. Although useful for the study of neuromuscular and visceral reactions and of changes in the volume and composition of the blood, experience has shown that—the problems of infliction of pain and utilization of dynamic apparatus quite aside—the cardiovascular responses may be even more complicated and difficult to interpret in unanesthetized than in properly anesthetized dogs. The dog is an animal which responds with tremendous variations in heart rate and blood pressure to trivial auditory, visual and surface stimuli, as well as to psychic influences. The latter may be minimized by training, but, despite claims to the contrary, cannot be eliminated entirely for studies of cardiovascular problems. The best criterion of normality in trained dogs is the persistence of their natural phasic sinus arrhythmia. However, during the course of a fairly rapid hemorrhage, which can be created painlessly under initial basal circumstances, intense psychic stimulation, manifested by movements or even struggle, seems to be caused by the cerebral anemia. The tremendous increase in respiratory rate and depth, exceeding in degree anything encountered in man, creates such an interference with the dynamics of the circulation that conclusions of consequence can rarely be drawn. While decerebration has its uses in cats, it probably causes even greater disturbances of the circulation than anesthesia, in dogs.

Since anesthesia is unavoidable in many types of experiments, and it is indeed questionable whether its elimination is desirable in shock experiments, it is important to utilize a type which interferes least with natural cardiovascular and nervous reactions. It is also necessary to comprehend the directions in which the anesthetic may be expected to modify responses. Volatile anesthetics cannot be administered so as to maintain an even concentration in the air passages and blood and, therefore, invoke many actions which confuse the reactions of shock. The pressure pulses, particularly, show variations that are not recognized in electrocardiograms or mean arterial pressure tracings.

The ideal anesthesia is one given intravenously, which produces the

least deviation of natural reflexes and cardiac actions, which maintains an even anesthesia for hours without readministration and which, of course, does not induce circulatory states that can be confused with shock.

Among the many anesthetics, we no longer consider chloroform a suitable anesthetic for dogs. Chloralozane is satisfactory and particularly useful in experiments in which a slow heart and somewhat exaggerated vascular reflexes are an advantage. However, after numerous trials of many anesthetics during the past 12 years, our laboratory regards *morphine-barbiturate* anesthesia perfectly satisfactory for cardiodynamic studies, including shock and hemorrhage. The particular barbiturate used is chiefly determined by the duration of anesthesia required. For prolonged experiments, we prefer sodium barbital, since readministration is not required.

In view of the fact that certain experimenters believe that barbiturates cause significant depression of the heart, circulation and nervous reflexes, and some, indeed, claim that barbital *per se* causes shock (4), it is necessary to defend its choice as an anesthetic.

As emphasized by Tatum (242), the reactions of dogs to barbital depend not only on the dosage but on the rate of its administration. Furthermore, it is not sufficiently emphasized that many of the undesirable effects of barbital are eliminated when it is preceded by morphine. The routine practice in our laboratory is as follows: A preliminary subcutaneous injection of morphine sulfate (ca. 3 mgm./kilo) is given a half hour previously. About two-thirds of a calculated anesthetic dose (175 mgm./kilo) is injected into a small leg vein over a period of 5 minutes. The effects upon reflexes, state of relaxation, respiration, etc., are noted. Cerebral excitement or intensification of respiration is uncommon. As much of the remainder of the calculated dose is then administered, even more slowly, until the animal just fails to respond to cutaneous stimuli; but the tendon reflexes are exaggerated and the corneal reflex is retained. In other words, *the anesthetic dose is determined by biological standardization for each animal.*

In a dog thus anesthetized, respiratory movements are slightly depressed, an advantage in that normal CO_2 tensions are maintained in the alveoli and blood and the excessive breathing of unanesthetized dogs does not obscure dynamic changes of the circulation. Although barbiturates and morphine both depress gastro-intestinal movements and tonus, intestinal contractions do occur, and viscerosomatic as well as somato-visceral reflexes remain active (191). Bouhaert and Hey-

mans (34), among others, claim that barbital suppresses sinus and aortic pressor responses. This we cannot confirm; moreover, Heymans' first observations on carotid sinus reflexes were made partly in our laboratory (128) upon dogs anesthetized with barbital. Stimulation of pressor fibers (central vagus and sciatic nerves) are capable of yielding extraordinary increases in mean pressure. We frequently obtain elevations of mean pressures to 200–250 mm. Hg by stimulating the central ends of both vagus nerves. Normal blood pressure levels are maintained for 12–24 hours when no experiments are carried out and the dog's temperature and water-balance are maintained. Indeed, such dogs recover completely and, subsequently, display no abnormality of action or behavior.

On the other hand, it cannot be claimed that barbital is without effect on the cardiovascular system or the reactions that may occur in the unanesthetized state. Barbital does tend to increase heart rate through diminution of vagal tone (111). However, extreme doses (ca. 500–1100 mgm.) are required to abolish such tone completely. Given with morphine, a natural sinus arrhythmia often persists, but even when this does not occur, section of the vagus nerves or atropine causes an additional increase in heart rate. Nor are peripheral vagus pathways blocked; stimulation of the peripheral end causes slowing or standstill of the heart and, in the case of the left vagus, leads to various types of A-V block. These are yearly classroom demonstrations in our school. In fact, the greatest cardiac acceleration which follows use of morphine and barbital is never more than that which occurs when a quietly resting dog is aroused. Shock induced in such barbitalized dogs merely resembles that of persons whose heart rate and vascular system have been altered before a catastrophe by psychic stimulation, e.g., fear, anxiety, excitement, fright, etc. Nevertheless, experimenters must recognize that the early increase in heart rate and progressive acceleration are less than under natural conditions and may even be missed in barbitalized dogs. For their evaluation, chloralozane anesthesia is superior.

Despite the increase in heart rate occasioned by barbital, arterial pressures quickly readjust themselves at approximately normal levels. Since barbital has been observed to dilate visible vessels and those of perfused organs, this is generally attributed to compensatory vasodilatation. It is difficult to prove, however, that this is the chief, or even an important mechanism. To us, it seems more probable that the enlarging spleen (1, 2, 51, 108, 115, 227) stores blood, reduces the venous return and keeps the minute output of the heart practically constant, despite

the increase in heart rate. Creation of such a greater reservoir may constitute an arrangement by which reduced blood volumes during shock and hemorrhage are better compensated through splenic contraction. Amytal also causes some hemodilution (1, 2, 74). Through these alterations, barbiturates should exert a protective rather than a deleterious influence in the production of shock. Seevers and Tatum (228) reported some congestion of viscera, and especially of the brain, after use of barbital; but it must be stressed that this occurred after chronic barbiturate poisoning and not after a single anesthetic dose. Moreover, death was not due to shock, but generally to convulsions.

Equivalence of experimental conditions. In experimental studies of shock it has seemed important to duplicate in anesthetized animals, as far as feasible, catastrophes that occur in man. The procedures employed in accordance with this aim include hemorrhage, crushing of muscles, bones and testes, infliction of burns, production of intestinal obstruction, exposure and manipulation of intestines, radiation of the abdomen, introduction of foreign material into the abdominal cavity, etc. It has been learned that all of these can cause shock, but that they do not invariably do so. Why this is true we do not know; some suggestions will be considered later. But when shock does eventuate as a result of identical procedures, different groups of investigators report different reactions. In order to evaluate to what extent this is due to the animal's resistance or susceptibility, the conduct of experiments must be scrutinized. It is important that all discoverable experimental variables be eliminated, or that their influence be assessed. Some of these may seemingly be of little importance but actually are determinants of variable results. For example, the changes of an animal's temperature may have a greater importance in the development of shock than has been commonly recognized. Fundamental studies (100, 208) seem to indicate that cutaneous blood can be mobilized to compensate for reduced blood volume only when the skin is kept moderately warm; the reactions of cooled and overheated animals differ markedly in physiological tests. While it is difficult to obtain conclusive evidence, Blalock³ recently recorded his impressions that shock may be produced more readily if animals are kept warm than if they are allowed to cool. It is a coincidence that Werle and I (266) recently formed similar impressions.

In the hope that use of more drastic measures may cause experimental shock with greater certainty, other experimental procedures have been

³ Address before the American Heart Association, Cleveland, June, 1941.

tried, such as prolonged hyperthermia or freezing, placement of ice-water in the abdominal cavity, rectum or intestinal loops, cauterization or traumatization of the intestines or liver, vigorous massage of the stomach, etc. Such experimentation may yield useful information, but the results must be applied to the broad problem of shock with reserve and caution.

Our duty as experimentalists is not concluded by producing shock and studying, as far as methods allow, the circulatory changes that take place. We must modify experiments cleverly in order to test the validity or unsoundness of numerous clues. In so doing, nerves must be cut and stimulated, important arteries or veins need to be sacrificed for insertion of proper cannulae, flowmeters or pressure recorders, etc. The plan of experimentation may even require opening of the chest or abdomen, manipulation of viscera, placement of oncometers, etc. All of these acts themselves modify the delicate balance of the circulation and its labile reflex control far more than the recording mercury manometer reveals.

Artificial respiration, opening of the thorax and exposure of the heart are required only in particular types of cardiodynamic experiments on shock. In the use of artificial ventilation, acapnia and chemical changes in the blood are largely avoided if artificial respiration is reduced to the point at which mild natural breathing just continues. Presence of Hering-Breuer reflexes, indicated by contraction of the diaphragm with each deflation of the lungs, is also a good criterion of a well adjusted artificial respiration. The degree to which pulmonary blood flow is impaired by the positive intrapulmonary pressures can easily be checked, from time to time, by noting the extent to which arterial pressure rises during momentary discontinuance of artificial respiration. The change should not be great. If low artificial respiration fails to maintain adequate oxygenation, as indicated by the darkening of arterial blood or attempts of the animal at deep spontaneous breathing, it is better to mix a little oxygen with respired air, than to increase the rate and depth of lung ventilation. I always feel that young investigators in the process of training have made considerable progress when they have mastered the art of inducing anesthesia and of giving artificial respiration properly. Many experimenters unfortunately have never learned this, or do not choose to give attention to it.

Opening of the chest, despite the avoidance of significant hemorrhage and of cutting numerous intercostal nerves, causes immediate changes in the pressure pulses which cannot be differentiated from the initial stages of hemorrhage or of shock due to trauma. The reasons remain

unsolved and deserve further study. These deleterious effects can be neutralized by maintaining a continuous slow infusion of warm saline and by slight compression of the thoracic aorta above the diaphragm, i.e., by use of the "controlled circulation preparation" (Wiggers, 260). These expedients which have proved so useful for experiments limited to cardiodynamic problems are not permissible in studies of the shock problem owing to the hemodilution incurred. Unless other expedients can be found which restore normal cardiodynamic conditions in open chest experiments, it must be recognized that we are starting with hemodynamic changes that have all the earmarks of the incipient stages of shock. However, there is one difference. If the anesthesia, artificial respiration, and temperature of the animal are properly maintained, the condition does not generally progress as in traumatic shock; on the contrary, it may improve after several hours. Consequently, significant information can still be gained regarding the changes produced by superimposed insults.

The proper evaluation of an animal's circulatory condition prior to use of shock-producing agencies cannot be over-emphasized. Since the qualitative and quantitative characteristics of *central arterial pressure pulses* are of the greatest service in forming such judgments, it is regrettable that their optical registration has been so slow in superseding recordings of mean arterial pressure.

In testing the mechanisms concerned in the initiation and progression of shock, it has often proved necessary to adopt methods for causing circulatory failure that bear no resemblance to those employed by Nature. The purpose of such experiments has frequently been misunderstood, with the result that experimenters have been criticized, and have criticized each other, as to the unapplicability of results so obtained. The category of procedures to which we refer includes prolonged strong stimulation of afferent nerves, injections of epinephrine, fat emulsions, muscle and tissue extracts, blood and serum from dogs in shock, administration of peptone, histamine, potassium and other agents; complete obstruction and subsequent release of blood flow from certain territories; also compression of the vena cava or aorta, pericardial effusions, occlusion of pulmonary vessels, reduction of plasma volume by plasmapheresis, prolonged intensive diuresis, etc.

In interpreting the factors responsible for shock, it is important that the fundamental purpose of such experiments be kept in mind. Most of these procedures may not have reduplicated the phenomena of shock produced in man, but they have proved useful in giving or testing clues

as to the nature of the disorder (see recent discussion by author, 266). However, it is equally obvious that the sequence of changes found in such clever experiments cannot be transferred directly to shock produced by more natural methods.

CRITERIA OF SHOCK. In order to study shock, criteria are necessary to determine when it exists. In such decision, the experimenter who works with anesthetized animals is deprived of many signs and symptoms that aid in its clinical diagnosis, e.g., changes in facial expression, motor and sensory depression, the appearance of the skin, sweating, etc. However, it is questionable whether shock, in the commonly accepted usage of the term, can be differentiated even clinically from pseudo-shock in its incipient stage. Blood pressure changes are generally admitted to be unreliable during the early stages; pressures may be normal or even high while forces which initiate shock are operating; or, they may be low, as a result of conditions not considered to be shock. The changes in heart rate are without value both in animals or man (144). Even the characteristic form of arterial pressure pulses and changes in venous pressure during early stages of shock are reduplicated by many other conditions.

During the progressive stage, the evidences become clearer. The best criterion is the coexistence of a progressive decline in central venous pressures, in cardiac output, and in arterial pressures. The first two are difficult to measure routinely, but in animal experiments qualitative and quantitative changes in the central arterial pressure pulses permit judgment as to changes in cardiac output and the fullness of the arterial system. With the introduction of optical manometers applicable to selected surgical cases, the direct registration of pressure pulses should prove increasingly more valuable. Preliminary studies by Volpitto, Woodbury and Hamilton (247) clearly indicate that the changes which occur during progressive stages of shock in man resemble those found in experimental animals. Lacking such facilities, the progressive fall in arterial pressures and the decrease in pulse pressure remain the most satisfactory general criterion during the progressive stage of shock.⁴

According to some investigators (for reference see Moon, 176; Scudder, 226), other signs, such as demonstrable reduction in blood volume and hemoconcentration, may even precede the decline in venous and arterial

⁴ It should scarcely require re-emphasis that pulse pressure of experimental animals cannot be measured by the recording mercury manometer. Unfortunately, this elementary fact is still not appreciated by many otherwise able investigators.

pressures. According to others (for references see Blalock, 29 and Harkins, 117), hemodilution sometimes persists when irreversible stages have been reached. This, at least, is true; certainly not every human subject with hemoconcentration or reduction in blood volume develops shock. Dominated by current emphasis on the importance of blood studies, erroneous diagnoses of clinical and experimental shock are unquestionably being made on the basis of hemoconcentration. Beneficial effects are claimed for various forms of therapy in instances in which it was never shown that the subjects were in a state of shock which would have proved fatal without treatment. Hemoconcentration is a valuable sign, only when it occurs in conjunction with the dynamic criteria mentioned above.

Since one of the outstanding characteristics of shock is its fatal tendency, shock, in a highly restricted sense, can perhaps not be said to exist until a stage of irreversible circulatory failure has developed. We have substantiated observations of others that blood pressures may be kept at very low levels (50-60 mm. Hg) and cardiac output may remain materially reduced for 2-3 hours by bleeding, yet recovery occurs in some animals when blood is restored to the circulation. Have such animals been rescued from shock, or did a state of shock never exist? We incline to the latter view. It is apparently possible to have all the signs without a state of shock.

Investigators frequently stress the advent of death as evidence that shock has been produced. However, such an accident, without critical relation to the previous course of events, may be without value. Sudden death through respiratory failure is an occasional accident in anesthetized animals during any state of hypotension and must not be confused with death due to shock. Such respiratory failure may be due to a neurogenic factor. Thus Mann (163) found that deep anesthesia may abolish all respiratory reflexes except inhibitory ones so that stimulation of an afferent nerve such as the vagus causes a permanent apnea which is followed by a rapid decline in blood pressure and cardiac failure. We have had similar experiences in deeply anesthetized animals subjected to critical hemorrhages, but always found it possible to revive them by prompt use of artificial respiration and small transfusions.

Autopsy findings. Within recent years, evidence has been accumulating that shock due to various causes shows distinctive changes at autopsy. According to Moon, "marked diffuse congestion of capillaries and venules in visceral areas, especially in the lungs, liver, kidneys and serous and mucous surfaces; edema, effusions into serous cavities and

capillary hemorrhages," are present in all forms of shock except hemorrhage, in which pallor and dryness occur. Curiously, the vessels of somatic structures do not appear to be affected.

However, differences of opinion still exist with regard to the uniformity with which such pathological changes develop in unquestionable death from shock.

The following tabulation gives a partial list of the variations presented in representative pathological reports:

| | |
|---|---|
| Blalock (27) | Capillary congestion, small hemorrhages, early |
| Brooks and Blalock (37) | necrosis in small intestines after hemorrhage. |
| Cornioley and Kotzareff (quoted by Moon) | Hemorrhages into stomach, liver, lungs, heart, pericardium, etc., after traumatic shock, in rabbits. |
| Davis (60) | No splanchnic congestion after hemorrhage or |
| Davis et al. (61) | traumatic shock—pulmonary edema, cardiac dilatation, liver necrosis after adrenalin shock— splanchnic congestion, etc., after histamine. |
| Erlanger and Gasser (76) | No ascites—marked congestion and hemorrhages of intestinal mucosa—increased blood-tinged intestinal secretions—liver not congested—all, in various types of shock studied. |
| Freeman et al. (93) | Congestion and edema of viscera after hemorrhage. |
| Klemperer et al. (146) | Focal or fused hemorrhagic lesions in stomach and gut after epinephrine. |
| Mann (162) | Primary dilatation of splanchnic vessels; later, congestion, following intestinal manipulation. |
| O'Shaughnessy and Slome (190) | Pallor of abdominal viscera and omentum after traumatic shock—congestion and edema after histamine. |
| Simonart (230a) | General dilatation of capillaries and plasma, transudation after burns and histamine—none in traumatic shock. |
| Keeley et al. (143) | Ischemia of intestines, no hemorrhages—lungs dry and slightly congested, spleen and liver firm after thermal trauma. |
| Turek (246) | Purplish discoloration of gut and venous conges- tion of abdominal viscera, in toxic shock. |

| | |
|------------------------------------|--|
| Wallace, Fraser and Drummond (248) | Report that surgeons performing abdominal operations in cases of wound shock noted only pallor of intestines—venous congestion never observed. |
| Whipple et al. (252) | Congestion of gastro-intestinal tract, liver, kidneys and lungs—engorgement and swelling of mucosa after toxemic shock. |
| Zwemer and Seudder (269) | Engorgement of intestines, lungs, etc., after trauma. Bloody peritoneal fluid, subserosal ecchymoses, swollen kidneys, petechial hemorrhages in lungs after intestinal manipulation. |

The spleen has been reported both as large and congested (76, 122, 252, 269) and as firm, contracted, anemic and dry (143, 157, 176, 177, 223).

While the pathological changes found at autopsy should be more carefully noted by experimenters, it does not seem that the picture is sufficiently constant to permit verification of a diagnosis of shock by post-mortem studies.

THE REDUCED VENOUS RETURN AND DECREASED CARDIAC OUTPUT. Reduction in the volume of blood returned to the heart is the keystone of all modern conceptions of shock. Actually the idea is a very old one. It dates from the classical experiments of Goltz (1864) (103) who demonstrated that sharp taps on the abdomen of a frog caused the heart to stop and that, upon resumption of its beat, little blood was expelled, because it had accumulated in the abdominal vessels and could apparently not be driven back to the heart. It was supported by observations attributed to Salathé (220) that acute circulatory failure can be produced in rabbits by suddenly tilting them from a horizontal to a vertical position. This is fundamentally similar to the "gravity shock" which occurs in certain individuals on standing. (For review of recent work, see V. E. Hall, 113.)

Splanchnic congestion was included in descriptions of shock-like states by Groeninger (110), Fischer (82), Crile (55), and Romberg and Pässler (213). All sensed correctly that the consequent reduction in cardiac output is the cause of the small, thready pulse. However, all attributed the decline of arterial pressure to diminished resistance in the splanchnic arterioles. That reduced venous return and decreased cardiac output are likewise responsible for the decline of arterial pressure was first suggested and largely developed by Y. Henderson (122, 123, 124, 125).

This important contribution of Y. Henderson to the analysis of

the dynamics of shock is generally overlooked in reviews on shock,—credit for the *idea* being sometimes given to subsequent investigators. This is in part due to his concurrent emphasis of acapnia as the factor responsible for respiratory failure, tachycardia, increased cardiac tonus, as well as failure of venous return in circulatory failure. With abandonment of the acapnia hypothesis, this clear and fundamental analysis of the cardiodynamics of shock should be given the credit it deserves. As early as 1908 (122), Henderson showed clearly that reduction of venous return reduced the stroke volume and how it did so (see Ref. 122, Fig. 1, p. 146). He further showed that excessive pulmonary ventilation decreased the diastolic filling and systolic discharge of the ventricles and, since both could be increased temporarily by saline infusion, the proper inference followed that reduced filling resulted in decreased output and fall in blood pressure. A fair evaluation of Y. Henderson's numerous contributions indicate a keen insight into the dynamics of the circulation responsible for the low pressures of shock. However, a careful scrutiny of *all* his published work fails to reveal experimental data which prove that such a cardiac mechanism operates in experimental shock due to trauma, intestinal manipulation or the other numerous ways in which shock has been produced. The credit for producing actual evidence that cardiac output—estimated by gasometric methods—does decrease in experimental shock produced in many different ways would seem to belong to Blalock and his associates (26, 140).

It is true that there have been—and still continue to appear—reports purporting to demonstrate reduction in venous return by observations on venous pressures. Clinicians always stressed the collapsed or invisible veins of the skin and the feeble jugular pulsations. However, decreased venous pressures cannot be judged from the appearance of surface veins (cf. Eyster, 78). Many experimenters (39, 55, 161, 178, 225) have reported decreased peripheral venous pressures in shock. However, Penfield (193) observed an increase, while Erlanger, Gesell and Gasser (77), and Bellis and Wangenstein (23) report only insignificant changes in jugular and inferior caval pressures, respectively. We must insist on clearer thinking with respect to the significance of venous pressure measurements. The pressure as measured in peripheral veins is no more an index of right auricular pressure than a blood sample from a peripheral vein is a criterion of mixed venous blood. That venous pressures measured elsewhere than in or near the right auricle are useless in estimating changes in venous return during shock is particularly clear

as a result of observations of Blalock and his associates (30, 32) that the reduction of flow from various regions is by no means equal during profound shock or hemorrhage. Furthermore, as Henderson and Barringer (123) pointed out, ventricular filling in the closed chest is not determined by the actual pressure in the central veins but by the difference between intra-auricular and intrathoracic pressure, i.e., by the "*effective venous pressure*" (cf. also Wiggers, 256). Since the tonus of respiratory muscles, upon which the magnitude of negative intrathoracic pressure depends, may conceivably alter in shock, it is of great importance to measure *effective right auricular* rather than actual central venous pressure.

In accordance with such reasoning, the writer in 1918 (257) believed he had made a significant contribution in showing that effective venous pressure, measured differentially, falls after infliction of trauma and intestinal exposure. I am now worried lest the original venous pressures may have been elevated by use of ether. Further, such observations do not permit one to estimate to the extent to which such a decrease is due to changes in intrathoracic or venous pressures. It is certainly more informative to record intrathoracic and central venous pressures separately. Such observations during the progress of shock are still needed in order to complete our picture of the dynamic phenomena. In addition, it must be kept in mind that while central venous pressures ordinarily reflect changes in volume of returning blood, such pressures are determined by the balance between rate of return flow, the size of veins and the rate with which blood is pumped away by the right heart. (For recent observations cf. Holt, 131.)

Finally, the majority of investigators, in efforts to fit experimental facts together, have assumed that venous return and cardiac output are necessarily decreased in proportion to a measured reduction in total blood volume. This is certainly not true in early stages of hemorrhage, for Meek and Eyster (171) showed that hemorrhage equal to 2.1 per cent of the body weight, or 28 per cent of the estimated blood volume, may not decrease cardiac output at all, presumably because reserve blood from the spleen, liver, skin and, perhaps, the lungs is placed in active circulation. Unquestionably, similar compensatory reactions occur during shock. *We may conclude:* Our current view that reduced venous return and decreased effective venous pressures are significant factors in the initiation and early progression of the circulatory failure in shock is strongly suggested by experimental and clinical observations; but it could be supported by more crucial experiments. Whether the

progressive decrease in venous pressures is the ultimate cause of irreversible circulatory failure or whether other precipitating factors enter, has not yet been demonstrated experimentally (cf. page 109).

THE INITIATING AND CONSEQUENTIAL MECHANISMS OF SHOCK. A satisfactory explanation of the initial reduction of the venous return would go a long way in establishing the cause of shock. Two fundamental ideas dominate the current literature, viz., 1, that the circulating volume is reduced by hemorrhage or local loss of plasma, and 2, that blood stagnates in capillaries and is thus removed from effective circulation. These conceptions are not mutually exclusive; in many instances the chief arguments revolve about the question which is cause and which, effect.

As Harkins (117) has properly emphasized, an initiating factor is the cause of shock, in the same sense that the diphtheria bacillus is the cause of diphtheria. However, in both cases, the progress of the disorder and the death that frequently occurs are consequences of such a cause. Indeed, death may be prevented, even when a causative agent persists, if the secondary consequences can be avoided or treated. Hence, the consequential effects which lead to irreversible circulatory failure are equally as important as the initial cause of shock. Unfortunately, it is not easy to separate causes and effects in various forms of shock; the factor which is a cause under one condition may prove to be an effect under other circumstances. It is becoming increasingly obvious that attempts to attribute shock to a common initiating factor, must probably be abandoned.

It cannot be disputed that significant decrease in blood volume is an important primary factor in hemorrhage and in the severe dehydration that follows protracted vomiting, prolonged diarrhea, excessive loss of gastro-intestinal secretions or their loss by drainage, loss of serum from wounds and burns, formation of large inflammatory exudates, etc. Blalock (29) and Harkins (117) have recently reviewed the overwhelming evidence that local loss of fluid at the site of trauma or from extensive burns is the important factor in the initial reduction of cardiac output. Such loss of fluid, if rapid and great, may, as in large hemorrhages, prove fatal due to respiratory failure. It can be postulated, that when the circulation is barely maintained for hours with the aid of compensatory mechanisms, this leads more gradually to general asphyxiation. Capillary stasis and increase in capillary permeability could be one of the consequences. The increase in capillary capacity reduces the effective circulating volume, and loss of plasma decreases the actual blood volume

further. A vicious cycle is obviously established. It is apparent that, according to this view, capillary stasis and generalized loss of plasma are consequences, not an initial cause, of shock.

However, an initial loss of fluid, sufficient to affect venous return significantly, is not apparent in many infectious or toxemic conditions which lead to shock. Moreover, since evidence can also be cited that toxic agents are absorbed when tissues are injured, and that nervous reflexes may affect the peripheral circulation, the view developed that, in such cases, changes in capillary capacity and permeability constitute the primary factor, and that changes in blood volume are secondary.

FACTORS RESPONSIBLE FOR CAPILLARY STASIS AND INCREASED PERMEABILITY. Since changes in capillary capacity and permeability occur either as primary or secondary phenomena in many instances of shock we shall advance our analysis of the peripheral circulatory failure by examining systematically the mechanisms by which such changes could be brought about.

Primary arteriolar dilatation. The arterioles are the terminal stop-cocks of the arterial tree which regulate the volume flow of blood from the arteries into the capillaries. Ever since the pioneer experiments of Claude Bernard on the salivary glands, physiological evidence has supported the thesis that arteriolar dilatation increases capillary pressure and volume. Unused capillaries become patent; the filtration and flow of lymph are augmented (for recent work and references see Maurer (167)) and the organ or district affected increases in volume. This is the fundamental reaction in inflammatory hyperemia and is apparently the response of the frog's peripheral vessels to remote trauma (Zweifach, 268). As a result of such arteriolar dilatation, an increasing number of capillaries fill, and the venous flow from an organ at first increases; but, if widespread territories are involved, the arterial pressure declines and venous flow is reduced. Meanwhile, a volume of blood equal to that which remains in the capillaries is prevented from returning to the heart. It is not difficult to calculate that such an abstracted volume may be quite sizable (40, 127). It can be argued that such withdrawal occurs essentially from the arterial rather than the venous side and that total venous return may not be affected. However, the question cannot be settled by such *a priori* considerations because the regulation of the circulation, in intact mammals, is highly complicated. The direction and magnitude of the change in total venous return to the right auricle depends on the extent to which arterial pressures fall, on compensatory vasomotor or cardiac reactions and on translocation of fluid from various

blood reservoirs. Thus maximal dilatation of arterioles by nitrites leaves the total return flow and auricular pressures unaltered (258), whereas dilatation induced by histamine is said to reduce it in dogs, apparently because the hepatic venous flow is impeded (101).

The concept that primary arteriolar dilatation is an initiating agent in shock has been generally abandoned, largely because it had become so definitely associated with the theory of primary failure of the vasomotor center. It is not necessary to rehearse again the evidence which definitely proves that this is not an initiating factor (40, 42, 176, 201, 202). However, it is not compulsory to assume, particularly in toxemic forms of shock, that arteriolar dilatation is exclusively dependent on failure of a central or even a peripheral nervous mechanism; it could be caused by action of the same humoral agents which many believe to act on capillaries. Indeed, it is not improbable that, if any such agents exist, they might affect arterioles, capillaries and venules alike. Whether or not arteriolar dilatation occurs depends to some extent on the manner in which shock is produced. If the abdominal viscera are inspected shortly after a large hemorrhage or infliction of trauma, they are generally pale. By contrast, the rapid progressive reddening of intestines, mesentery and omentum which occurs when they are exposed and manipulated cannot have escaped surgeons or experimenters (162, 246). Capillary engorgement through vasodilatation is obvious; whether this is significant in reducing central venous pressure can be debated, and remains to be demonstrated.

The argument that primary arteriolar dilatation is excluded because, in shock, the decrease in cardiac output precedes the fall in blood pressure (Blalock, 29) is not a valid one. It is conceivable, and indeed probable, that primary dilatation, even in fairly extensive regions, could be compensated, *pari passu*, by cardiac acceleration and arteriolar constriction in other regions. Blalock's own experiments indicate a greater reduction of blood flow through limbs than elsewhere (30, 32). A similar compensation occurs very quickly in normal human subjects after inhalation of amyl nitrite; pressures are restored in 1 to 2 minutes, while the skin vessels obviously remain dilated. Compensatory constrictions might mobilize blood from the spleen (2, 12, 108, 115, 235), liver (15, 104, 142, 149, 187, 197), lungs (63, 130) and skin (13, 100, 187, 189, 207, 208), and this may be not merely sufficient to compensate for any local reduction in venous flow from organs involved but may even supply a larger return volume for the accelerated heart to pump.

In 1918, I (257) published arterial pressure pulses which seemed to indicate that reduction in peripheral resistance is a very early reaction

in shock due to intestinal manipulation and trauma. The intent of these inferences has apparently been misunderstood. It was never my belief that such reduction of peripheral resistance remains an important factor during the progressive decline of blood pressures but that, in types of shock studied, it might constitute an initial reaction which favors capillary stasis and reduction in effective circulatory volume. I am ready to grant, on the basis of broader experience, that the initial changes in the contour of pressure pulses may have another explanation, and that the evidence is not as crucial as I then believed. However, the changes in the form of the aortic pressure pulses then presented have never been controverted nor explained more satisfactorily by others. A study of changes in blood flow in the mesenteric arteries and portal vein correlated with alterations in the form of central pressure pulses is highly desirable during the early and progressive stages of shock. I hold no brief for this or any other conception as to how peripheral circulatory failure is initiated, but insist that it is inadvisable to cast aside too hastily the hypothesis of a primary visceral dilatation.

Primary arteriolar constriction. The conception that shock is initiated and maintained by virtue of an intense vasoconstriction had its origin in the clinic. It is difficult to assign priority to the idea; but, as is well known, it constituted the favored view among English clinicians from Mapother (165) to Malcolm (160). However, the clinical evidence and logic upon which this was based can no longer be regarded as satisfactory. The theory seemed to receive considerable support from experiments of Bainbridge and Trevan (9), of Erlanger and Gasser (76), and of Freeman et al. (89, 90, 91, 92, 93, 94), who demonstrated that reduction in blood volume, hemoconcentration and circulatory failure can be produced by prolonged administration of adrenalin or by compression of the aorta. The idea was given a real impetus by the often quoted experiments of Gesell (96, 97) who showed that, after hemorrhage or tissue abuse, the blood flow through the salivary glands and muscles decrease significantly before any fall of arterial pressure could be detected.

Since generalized arteriolar constriction *per se* reduces the capillary pressure and capacity in any territory, shrinks the organ affected and, in intact animals, often increases venous pressure and return (51), secondary mechanisms must exist to produce just the opposite effects characteristic of shock. Two possible mechanisms can be invoked:

1. The decreased capillary flow following an initial arteriolar constriction may cause an anoxic, asphyxial or ischemic action, which relaxes patent capillaries and opens up those ordinarily closed. If so,

capillary pressure would rise and filtration through the walls increase. Blood is not merely sequestered, but greater plasma leakage develops. Such stagnation of blood has at various times been placed in muscles (42, 234), in the intestinal mucosa (76, 126), in the liver (139), and, in addition, in the lungs (176). The concept accepts the findings of Krogh and his school (150) that capillaries possess the power of independent contractility and tonus. It is supported by observations of Landis (152) that temporary compression of afferent arterioles causes dilatation of the capillaries and allows the free passage of dyes. But it ignores equally valid observations of Clark and Clark (47) that capillaries change their size only in a passive manner.

2. If, as Zweifach (267) has recently claimed, the main nutritional capillaries are not directly interposed between the arteries and veins but represent a series of shunts, an extension of arteriolar constriction to the main A-V capillary would divert blood into the *real capillaries* causing the expansion of those in use and the opening of others not previously in action. Similar capillary filling may occur mechanically when larger arterio-venous shunts shut down (46) or when a second set of arterioles—as in the liver or kidney—constrict. This may explain the prompt increase in the volume of intestines or paws which has been reported after small doses of adrenalin (100).

Summarizing, it is possible, from a dynamic viewpoint, to postulate capillary stagnation after arteriolar constriction either on the basis of a passive process or of an active relaxation of the capillaries themselves.

Before we examine the evidence for and against the vasoconstriction theory it may be well to state that the theory has been given at least two separate interpretations. The more conservative hypothesis considers the original vasoconstriction as a compensatory mechanism which sustains arterial pressure and diverts blood to more vital organs, such as the heart and brain. But, if it persists until arterial pressures begin to decline it helps to throttle the blood supply generally, causing capillary stasis and loss of plasma. Such a conception which assigns a secondary evil effect to the compensatory constriction must be differentiated from the hypothesis, originated by Erlanger and associates and recently revived by Freeman (89, 90, 91, 92, 93, 94), that vasoconstriction is the initiating factor. According to Freeman, any overactivity of the sympathetic system or the adrenal medulla, of reflex or psychic origin, leads primarily to impairment of blood supply, induces capillary stasis and loss of plasma, and thus a reduction in circulatory volume and shock. Much of the experimental evidence originally supporting this hypothesis

was of a circumstantial nature. Experiments reported in 1933 (89) indicated that prolonged intravenous infusions of adrenalin in doses of 0.006 mgm./kilo/min. resulted in a reduction of the blood volume of cats, but no evidence was included that shock eventuated. Hamlin and Gregersen (114), using doses of 0.035 mgm./kilo/min., on the contrary, found an increase in blood volume of normal and sympathectomized cats. v. Prohaska, Harms and Dragstedt (203) were unable to produce shock in dogs by continuous administration of adrenalin for two weeks in doses of 0.0009 to 0.003 mgm./kilo/min. Recently, Freeman and his associates (91) were able to cause not only a reduction in blood volume but a decline of arterial pressure to shock levels by injection of adrenalin into atropinized, unanesthetized dogs at a rate of 0.0034 to 0.0164 mgm./kilo/min. for $\frac{1}{2}$ to $1\frac{1}{2}$ hours. Apparently, the subject merits further study. It still needs to be demonstrated that capillary congestion and increased permeability precede the reduction in blood volume and pressure and that the circulatory failure was not due to deleterious action of such prolonged injections on the heart.

The evidence for vasoconstriction. In order to implicate vasoconstriction as the factor responsible for the capillary engorgement, it is necessary to have substantial evidence 1, that arteriolar constriction occurs in the regions in which such capillary damage is most outstanding (e.g., in the intestines); 2, that it is sufficiently prolonged and intense to cause such capillary damage. In my judgment, neither has been demonstrated. Intense pallor of the skin is one of the conspicuous and persistent features of shock in man and, unquestionably, is due to intense arteriolar constriction. Good evidence exists, both in animals (179, 86, 216) and in man (94), that the volumes of limbs and their blood flow decrease materially during shock; further that the oxygen content of cutaneous venous blood is decreased and that the A-V oxygen difference is greater (6, 30, 122). It is remarkable, however, that despite such intense constriction and retardation of flow, cutaneous and somatic tissues do not exhibit any postmortem evidence of capillary damage (176). The facts do not fit together.

We may briefly examine the more direct experimental evidence which is so frequently quoted in support of the vasoconstrictor hypothesis. In 1909, Seelig and Lyon (225) found that the flow from a femoral vein measured for 5 second intervals decreased during shock, but no evidence was presented that this was not due to the steadily declining arterial pressures. The augmentation of flow which followed section of a sciatic nerve merely proved that vessels were still influenced by vasomotor

nerves but, of course, gave no evidence that the intensity of constriction exceeded the normal. In 1914, Mann (161) reported that normally innervated vessels in the tongues of dogs, ears of rabbits, and paws of kittens were constricted during shock in comparison with denervated vessels of an opposite side. Meek and Eyster (171) observed constriction of ear vessels during hemorrhage which led to *collapse of capillaries and venules*. Neither of these observations gives any support to the conception that vasoconstriction leads to capillary engorgement. Gesell in 1918 (96) demonstrated clearly that blood flow decreased markedly in the salivary gland, early in hemorrhage and after remote injuries to tissues; but the rest of his paper is pure speculation. Subsequently, Gesell and Moyle (97) demonstrated that the venous flow from a leg vein decreased during hemorrhage. Stagnation, edema or other deleterious influences in the glands or muscles were certainly not demonstrated.

In 1911 Cope (50), under my guidance, measured the rate of pressure decline in a temporarily occluded femoral artery, the collaterals of which had been ligated. He found an increased resistance in the leg vessels during early stages of hemorrhage and a decrease during the terminal stage. The method was again employed in 1922 by Forward and Perme (83) who found highly variable changes in resistance during shock, but, in general, they seemed to be in the direction of a decrease. Both of these investigators were careful in pointing out that changes in resistance so determined are not solely due to vasomotor changes but, in part, to changing venous pressure, blood viscosity, etc.

In 1912 Bartlett (16), under Erlanger's guidance, measured the rate at which a small volume of saline (ca. 2-5 cc.) under high pressure flowed into a leg artery "temporarily isolated from the circulation"—except for presence of numerous collaterals. Since such a small quantity of saline could obviously not reach all the arterioles and capillaries of the leg, the flow of the animal's own blood through these vessels was presumably determined. Hence changes in viscosity and variable collateral flow were perhaps not ruled out as completely as assumed. By this method, Bartlett found an increased rate of flow through the leg during early stages of shock. Erlanger, Gesell and Gasser (77) refined the method somewhat, and found, on the contrary, that the flow decreased through vessels of the limb and internal organs until late stages of shock. Similar results were apparently obtained by McKen Cattell (44).

Perfusion of a hind-limb, a kidney or an inferior mesenteric vessel by larger volumes of saline solutions were carried out by several investigators. Such perfusions have the advantage that a solution of constant viscosity is being used, but the disadvantage that edema develops and

the flow decreases progressively through increasing extravascular pressures. Furthermore, the rôle of collateral circulations, which can increase rapidly after ischemia (71), is not evaluated. Using such methods, Morrison and Hooker (178) and Penfield (193) reported results which indicated that the flow increases as shock develops. In 1914, Pilcher and Sollmann (199) perfused the spleen, separated from the general circulation, but left in connection with the central nervous system, with saline solution. They reported an initial increase in resistance and a decrease during terminal stages of hemorrhage. It is questionable, however, whether the dog's spleen is a good test organ for studying vasomotor reactions, for it is very difficult to discount effects due to contraction of its muscular trabeculae. In any event, constriction of splenic vessels is certainly not followed by stasis of blood in capillaries and sinusoids, as demanded by the vasoconstriction hypothesis. Moreover, constriction of splenic vessels increases venous return and circulating volume. Recently the Mayo investigators (10), using the thermostromuhr, reported rapid and marked reduction in femoral arterial flow while blood pressure still remained elevated during early stages of shock.

Summarizing, 1, a number of investigations strongly indicate that arterioles of certain regions, particularly the limbs, salivary glands and spleen, constrict during shock, but this conclusion is by no means unanimous; 2, those who report occurrence of vasoconstriction have never described coincident or sequential capillary damage in regions studied, and 3, the organs selected for studying such vasomotor change may not be representative of changes in the splanchnic region, in which the most outstanding capillary damage seems to occur. If primary vasoconstriction can cause reduction in blood volume and shock, other facts are difficult to understand:

1. Intense and prolonged stimulation of pressor nerves, such as the sciatic or central vagus end, do not lead to shock or pathological changes in capillaries (42, 112, 161, 225, 258).

2. The somatic structures which undergo the most constriction fail to show signs of capillary congestion, edema and hemorrhage at autopsy.

3. The fact that stimulation of pressor nerves or injection of adrenalin (88), neosyneplirin or related substances still cause substantial elevations of pressure during the course of shock strongly suggests that the arterioles are far from maximally constricted, as is sometimes claimed.

4. Prolonged anoxia of a degree far exceeding that which is probable after adrenalin causes serious symptoms in animals and man, but not those of shock (265).

To summarize, a critical examination of all experimental results sug-

gests that vasoconstriction may exist in certain territories of the body during shock, but there is no substantial evidence that it is sufficiently generalized or intense enough to cause the capillary damage which the hypothesis requires.

It remains to interpret the fact that cutaneous vessels are obviously constricted during shock. In the first place, it should be kept in mind that such cutaneous constriction occurs as a concomitant of many reactions in man which do not eventuate in shock, e.g., in gastric, biliary and renal colic, during acute gastric upsets, as a result of prolonged standing, preceding syncope due to noxious influences and emotional reactions. In the second place, abundant experimental evidence exists that clamping of all limb vessels affects arterial pressure and calculated total resistance, scarcely, if at all (18, 262). On the other hand, the subpapillary plexus of the skin represents a large blood depot from which considerable volumes may be pressed into internal circuits by vasoconstriction. Thus, injection of small doses of adrenalin or cooling decreases the blood content of the skin significantly when an animal is warm (100, 207). Consequently, the purpose of the intense cutaneous constriction during and even preceding shock is not to help maintain arterial pressure by increasing total resistance, but to inaugurate a compensatory and perhaps an anticipatory reaction by virtue of which larger blood volumes are shifted internally for circulation through viscera (and muscles?).

Primary injury and dilatation of capillaries. Space is lacking to review the vast amount of experimental work which has demonstrated that many foreign substances or those extracted from body tissues cause, upon intravenous injection, circulatory changes which resemble those of shock in many respects. They include, Heidenhain's "lymphagogues of the first class," decomposition products and extracts of the intestinal mucosa, liver, traumatized muscle, etc., traumatized red cells (196), some bacterial toxins and products of inflammation (54, 173, 209), snake venoms, histamine, acetylcholine, adenylic compounds, etc. (For references see 127, 150, 152, 176.) Most of these are unquestionably capillary poisons which alter the size, turgor and permeability of capillary walls, causing stasis, increase in lymph flow, and later, tissue edema. Moreover, various substances (liver, minced muscle, bile, etc.), placed in the peritoneal cavity, cause similar generalized effects (176). These and other experimental evidences suggested that as yet unidentified substances, formed during high intestinal obstruction, gastro-intestinal perforations, toxemia and infections, may act directly upon capillaries and so initiate shock.

The hypothesis of primary capillary injury has recently been brought into prominence through the pathological studies of Moon and his associates. We have already indicated that the existence of visceral congestion, edema, effusions and petechial hemorrhages at autopsy have not been universally confirmed by pathologists. It seems probable that they are limited to certain types of shock. Moreover, even in forms of shock in which they exist, specimens obtained at autopsy cannot furnish *proof* that capillary dilatation and leakage were primary rather than terminal changes. I know of no positive and direct evidence that incipient capillary dilatation and loss of plasma from capillaries accounts for the *initial decrease* in venous return. Even so powerful a capillary dilator as histamine apparently increases capillary permeability only temporarily (183) and leads to irreversible circulatory failure only when some other factor is superimposed (166, 206). The bold interpretation that the apparent prophylactic and curative properties of cortico-adrenal substances (cf. p. 107) are due to maintenance or restoration of capillary permeability is mere speculation. In view of the wide acceptance which this idea has gained, it is surprising that so few attempts have been made to test it experimentally, with particular attention to the time of the capillary changes and their disappearance after treatment. The nearest approach to such studies are the recent reports of Menkin (173) and of Freed and Lindner (87) who found that adrenal cortical extracts and corticosterone prevent capillary leakage of trypan blue induced by leukotaxin, but these investigations obtained contradictory results from use of desoxycorticosterone. The applicability of such results to changes in permeability of shock induced by other agents than leukotaxin remains to be established; but the investigations at least represent a start in the right direction. The question whether leukotaxin may not be identical with histamine is not fully settled (212), and we still lack conclusive evidence as to whether the permeability valence of capillaries is determined chiefly by stretching (151) or by changes in the intracellular cement (45).

The wet and congested appearance of viscera in animals and individuals who die from shock has been considered helpful in accounting for the reduction in circulatory volume, particularly in types of shock in which plasma loss is not otherwise obvious. According to Moon (176), "further search for the location of the 'lost blood' is unnecessary." However, quantitative relations between the amount of plasma lost and that remaining in the vascular system cannot be established by postmortem examinations. The weights of organs and comparison with average normals also do not suffice, but even such comparisons have only

occasionally been attempted. Hence, while the circulatory volume is unquestionably reduced in forms of shock in which an obvious large loss to the exterior or into tissues can be demonstrated, the question of a similar reduction in toxemic forms of shock deserves more critical study.

Plasma depletion through capillary leakage is also frequently inferred from changes in viscosity and specific gravity of blood, from increased red cell counts, hemoglobin or hematocrit readings. As Adolph et al. (2), among others, has pointed out, it is hazardous to use red cell counts or hematocrit readings in estimating hemoconcentration or hemodilution in shock and hemorrhage, since red cells may be mobilized from the spleen and other blood banks. Even the dye methods which have not been employed too extensively in the study of toxemic shock are not as conclusive as was originally hoped. (For limitations see 75, 98, 105, 188.) Two difficulties—failure of complete mixing in regions of reduced blood flow and rapid leakage of dyes through capillaries—render deductions hazardous during shocklike states.

Summarizing, the magnitude of fluid loss from the vascular system through leaking capillaries has not been satisfactorily established in toxemic forms of shock. Consequently, the possibility should be re-examined whether a change in the *effective* rather than the *actual* circulating volume may not be responsible for the reduced venous return in such conditions.

The problem of a toxic agent. If primary capillary dilatation is the result of a humoral factor, some form of toxic agent must exist. Of the many suspected agents, histamine has received the most thorough study. The earlier investigations of Dale, Laidlaw and Richards (56, 57, 58, 59) indicated that histamine injection, like shock, results in capillary stasis, transudation, increased gastro-intestinal secretions and lymph flow, and, later, edema, decrease in blood volume and reduction in arterial pressure. The conception that circulatory failure of traumatic shock is similarly due to absorption of histamine or a similar substance received what then seemed strong support. (For recent work see 7, 14, 65, 155, 157, 158.)

This was followed by a period during which experimental work began to question the guilt of histamine or, in fact, of any other toxic agent. Investigators are still not agreed as to whether the histamine content of blood is increased during shock (14, 48, 68, 69, 219), or whether blood from traumatized tissues contains more histamine (181, 182). Opinions are at variance as to whether any depressant substance is demonstrable (132, 190, 231, 232). If such toxic agents exist, transfusion of blood

from shocked dogs into normal animals may be expected to cause a fall of blood pressure in the recipient. Regarding the facts, experimenters are in apparently hopeless disagreement (3, 17, 22, 24, 49, 145, 148, 192, 218, 222, 253). However, the same situation exists regarding the humoral agents in hypertension; and it is questionable whether the problem can be settled by such injection studies.

Certain differences between circulatory reactions following injection of histamine and during shock have also been emphasized. According to Blalock (28), decrease in cardiac output precedes the decline of arterial pressure in shock, whereas the reverse is the case after histamine injections. The decrease in cardiac output is also much less in histamine shock (140). The bleeding volume required to cause death and the amount of blood that can be drained from organs after death is said to be much greater after histamine and other vasodilating agents than it is after shock induced by trauma (217). Partition of the return flow from various organs by ingenious methods led Blalock and Levy (32) to the conclusion that the venous flow is decreased proportionately from all parts of the body after histamine, but predominantly from the posterior extremity in shock following intestinal trauma and hemorrhage.

These differences are not necessarily of fundamental significance. The differing rates and portals of entry of injected and hypothetically absorbed histamine might account for these findings. When arterial and venous pressures and cardiometer tracings are recorded continuously during the course of a slow injection of histamine by femoral vein, reduction in venous pressure and stroke volume occur *pari passu* with, not after, the decline of blood pressure (personal observations with Werle).

To summarize, while histamine has not been finally excluded as the toxic agent, it is the consensus of opinion that it is not the agent concerned. If a toxic factor exists it is far more probable that a variety of agents rather than a common one is concerned. It may, as in the case of renin, require an activating agent and possible participation of an endocrine factor, which would explain the development of similar states of shock during cortico-adrenalin insufficiency.

Failure of a venopressor mechanism. The pressure component still available after blood has been driven through the capillaries into veins is the fundamental force which returns blood to the heart. Even with normal arterial pressures, its magnitude (ca. 16-17 mm. Hg), aided by a negative pressure of -3 to -4 mm. Hg in the thorax does not suffice to raise blood from the feet to the chest in the vertical position. It is

true that the hydrostatic pressure in the veins is counterbalanced by an equivalent hydrostatic pressure in the arteries, but, in order to become effective in returning blood to the heart in the vertical position capillary pressures in the feet would have to rise to values which would disorganize the exchange of fluid in the capillaries (Bazett, 20). It is obvious that some additional venopressor mechanism which helps the return of blood must exist. Its existence is also proved by the Rimpl experiment (211). This consists in suddenly clamping the pulmonary artery or stopping the heart, in which case pressures in the right auricle may rise to 9 to 10 mm. Hg, despite the fact that arteriolar pressure approaches zero.

The nature of this venopressor mechanism is still obscure, although authors of textbooks glibly repeat mechanisms not too surely demonstrated. The mechanisms suggested include *a*, extravascular support or compression of veins by an elastic tension of tissues and by the tonus or contraction of muscles; *b*, the pumping action of respiration (35), and *c*, active venomotor changes due to humoral or nervous actions (cf. Wiggers, 264; Bazett, 20).

We owe the suggestion that such a venopressor mechanism fails primarily in shock to Y. Henderson (122). It is no discredit that his conception as to the locus of the venopressor force appears to have changed in his search for this elusive mechanism. Originally, he postulated that lack of CO_2 relaxes small veins and smooth muscle around them, fills them with blood and thus reduces the effective circulating volume. Actual observations, that small veins increase in size during shock, are reported by a number of investigators (134, 164, 246, 268). With the evidence (5, 84, 133) that CO_2 relaxes muscles of the vascular system and gastro-intestinal tract, it proved equally easy to suggest that acapnial constriction hinders transmission of pressure from arterioles to veins and thus traps blood in the capillaries (124). Similar effects might be mediated by loss of tonus of the venomotor nerves, the presence of which seems to have been demonstrated for mesenteric (134, 135) as well as for superficial veins (11, 67, 70, 244). More recently, Henderson and his associates (125) reemphasized the conception that "the tissue pressure throughout the body combined with negative intrathoracic pressure . . . determines venous return." Starling (234) had previously made a similar suggestion. The conception holds that the longitudinal pull of tonically contracted muscle fibers causes a transverse pressure against blood vessels lying between them and thus prevents loss of intracapillary pressure and fluid.

The experimental evidence, recently summarized by Henderson (121),

consists in observations that pressure measured by a hypodermic needle connected to a manometer and thrust into the biceps normally equals 60 to 90 mm. H_2O , that this pressure decreases during anesthesia and operations, and that it approaches zero at death. Apropos are observations of Gesell and Moyle (97) who report a "palpable softening" of muscles in unanesthetized dogs and a recorded lengthening in resting muscles of some anesthetized dogs following hemorrhage. Other studies (21, 119, 168) indicate that persons with relatively low tissue pressures are more prone to faint on standing (gravitational shock). The problems of whether such simple procedures as have been used to measure tissue pressure are really reliable and whether such tension is chiefly due to tension of fascia (249) require further study. In studying the relation of intramyocardial pressures to coronary flow, Gregg and Eckstein (107) were compelled to conclude that reliable measurements of supporting pressure had not yet been achieved. Similar critical studies are necessary with respect to skeletal muscle pressures before much stock can be taken in the conclusions regarding changes in tissue pressures during shock.

More general types of evidence make it highly improbable 1, that muscular tonus represents more than a subsidiary mechanism for the return of blood, and 2, that failure of such return is due to absence of motor impulses necessary for maintenance of skeletal muscle tonus. Loss of such tonus as a result of deep anesthesia, administration of curare in animals or during paresis or myasthenia gravis in man does not eventuate in shock. Furthermore, according to Moon, the muscles are particularly free from the congestion, hemorrhages and edema so conspicuous in the viscera during shock. Perhaps cessation of intestinal movements or loss of tone in smooth muscle of viscera might be concerned, though this has not been proved. Finally, the Rimpler experiment succeeds in flaccid anesthetized animals whose chests have been opened, and we have recently obtained evidence that this venopressor force—whatever its cause—still operates to raise venous pressures during the terminal stage of shock. The force for returning blood is reduced, but by no means lost.

Summarizing, while subsidiary forces unquestionably aid return of venous blood, there is no good evidence for the attractive hypothesis that failure of a venopressor mechanism constitutes an important factor in the initiation or progression of peripheral circulatory failure.

Summary regarding initiating factors. Our analysis indicates that while it is easy to set up views as to how the reduction of venous return is initiated, the operation of none of the suggested mechanisms—arteriolar dilatation or constriction, capillary dilatation and changes in perme-

ability, failure of a venopressor force—has been proved by existing experimental evidence. On the other hand, obvious loss of blood (hemorrhage) or plasma (e.g., in traumatic shock) remains a clearly demonstrated initiating factor in many types of shock. Whether a similar loss occurs through capillary leakage in other forms of peripheral circulatory failure and by what mechanisms it is induced are problems that require further relentless research.

This conclusion may have a bearing on the recurring question, as to best procedures for producing experimental shock for purposes of scientific study. The writer's reaction would be that, lacking crucial evidence that the primary decrease in venous return is due to any factor other than loss of blood or plasma, it would be a step *toward* the standardization of procedures, if investigators more generally chose a method which is known to cause such a primary decrease in circulatory volume. Adoption of such a plan would probably increase the consistency of results with respect to the efficacy of therapeutic procedures, the evolving mechanisms, and the ultimate cause of irreversible circulatory failure.

Sustaining and Precipitating Mechanisms the Cause of Death. Following a suggestion by Gesell (96), writers continue to differentiate between initiating and sustaining factors in the causation of shock. The latter admirably discussed by Cannon (42), Blalock (29) and Harkins (117) include such developments as slowing of blood flow, lowering of metabolic rate, anoxia, acapnia (or hypercapnia), impaired renal excretion, alterations in acid-base balance, electrolytes of blood, etc. Concurrent dehydration through deprivation of water, profuse sweating, vomiting, diarrhea, lowering of body temperature, nervous reactions to fear and pain, prolonged anesthesia, concurrent effects of toxic products, infections, etc., may all contribute to a downward course. Lack of space prevents another review of their importance.

I have recently suggested (265, 266) that after a circulatory imbalance has been inaugurated by initiating factors, it still requires a precipitating mechanism (or agent) to convert it into a stage of irreversible circulatory failure. The precipitant may consist of a default in natural compensatory mechanisms or in the supervision of a new noxious factor.

That something breaks or defaults during the course of progressive circulatory failure and thus inaugurates an irreversible state is suggested by many facts gleaned partly from personal observations and partly from perusal of the literature on shock: 1. A laboratory procedure designed to produce experimental shock may result in only a moderate imbalance of the circulation which lasts for hours, when, quite suddenly,

a downward trend of blood pressure begins which leads speedily to a state of irreversible circulatory failure. 2. While shock which follows a progressive hemorrhage certainly starts with a decrease in circulating volume, inadequacy of venous return alone does not satisfactorily explain the terminal circulatory failure in all animals. Sometimes the central venous pressure even shows a premortal recovery. 3. Infusions of blood, plasma or even saline exert a highly beneficial effect during the early stages of shock, but they become ineffective or may even prove deleterious after an ill-defined non-reactive stage has been reached. 4. Identical procedures, standardized as far as individual laboratories are concerned, produce shock in some animals, but utterly fail to do so in others. More thought and experimental effort ought to be directed toward the cause of such resistance or refractoriness of animals and humans to shock. It is after all not strange that shock can be produced by catastrophies short of being fatal; it is, on the contrary, remarkable that the body may survive very drastic insults. In considering some of the factors which may be concerned in precipitating an irreversible circulatory state, we must again curb our desire to establish one dominant factor for all types of shock. A single prepotent factor may exist; but it is a better wager that a number are concerned.

The Time Factor; Ischemia; Irretrievable Capillary Damage. As long as blood pressure is maintained fairly well, the degree of vasoconstriction which exists throughout the course of shock is apparently not intense enough to reduce blood flow to damaging levels. Even with reduction in blood corpuscles, after considerable hemorrhage, no measurable anoxia may exist (99). On the other hand, abundant evidence supports the view that this may occur when the head of pressure in the aorta has become low. *A priori*, it seems probable that if such impairment of flow persists for a long time, it may have the same effect as a more temporary complete anemia. Penfield (193) adduced experimental evidence that the duration of low pressure is of importance in production of shock. However, it is surprising how well tissues or organs withstand a very low rate of blood flow before they cease to function or are unable to revive. If it were not for this factor, physiological perfusion experiments would never have reached the importance they have. Erlanger and Gasser (76) caused capillary damage and shock through ischemia produced by clamping of the aorta; but Quigley and Lindquist (204) and Parsons and Phemister (192) were not able to do so in unanesthetized animals. It should not be concluded too hastily that the differences were due to vital effects of anesthesia. The possibility that the intensity

of the ischemia was less owing to better collateral supply must be considered.

In our studies of hemorrhagic shock, Werle, Cosby, Wegria and I have, during the past year, studied many dogs that were kept at mean arterial pressures of 70 and 50 mm. Hg for 3 to 5 hours and others at 40 mm. Hg or less for shorter intervals. At the end of such periods of hypotension they were reinfused with all the blood (heparinized) lost by hemorrhage. Our experience coincides with that of others (e.g., 42, 162, 193) that many dogs recover from a hypotension of 50 mm. Hg maintained for 2 to 3½ hours, whereas few are more than temporarily benefited by reinfusion when pressures have been at 30 to 35 mm. Hg for 45 to 60 minutes. Two factors are apparently of dominant importance,—the extent to which aortic pressure has been reduced and its duration. Examination of the mucosa of the upper intestine invariably showed intensive congestion, edema and hemorrhages with free blood or blood-tinged fluid in the lumen of those dogs that failed to recover. In those that did recover the abdominal viscera were pale or pink externally and the mucosa of the intestine either appeared normal or only slightly cyanotic and swollen. Such observations may have a bearing on the different pathological descriptions of the viscera after shock summarized on page 86. More important, however, they stress the fact that extensive capillary damage occurs only after a marked hypotension has existed for a considerable time. It is obviously a consequence, not a cause of the hypotension. Furthermore, it is by no means demonstrated that the failure of reinfusion to restore and maintain normal arterial pressures in non-surviving dogs is solely or even largely due to accumulation of all the reinjected blood in the intestinal mucosa or its lumen. Such an inference requires additional experimental support.

On the basis of many physiological studies of capillary behavior (for reviews see 150, 152), the development of complete capillary damage would be most expected under conditions in which anoxic or toxic actions come into play. Marked anoxia unquestionably exists during prolonged periods of decreased blood flow, but it is merely an inference that capillary endothelium is irretrievably damaged thereby. The increased lymph formed during extreme anoxia (118, 167) must have been completely returned to the circulation *via* lymphatic ducts in those experiments in which organs are reported "dry" at autopsy. Furthermore, histamine, which certainly exerts a very toxic action on capillaries, producing engorgement, edema and even petechial hemorrhages, does

not necessarily lead to irreversible shock even when administered over long periods of time. Without any desire to minimize capillary damage, it is hazardous to infer that such damage is solely responsible for the failure of response to blood or plasma transfusions.

Cortico-adrenal influences. Within recent years, the rôle of the adrenal cortex and its hormones in determining the resistance to shock-producing agents has received considerable attention. Studies on the effects of adrenalectomy and injection of progressively more purified hormones by a host of investigators were originally undertaken to clarify the cause of death from Addison's disease. During the course of such studies, it became increasingly more apparent to many observers that, while the functions of many organs are affected by adrenalectomy, the animals die in a state of circulatory failure which resembles shock from other causes. Swingle and his co-workers (237, 238, 239, 240, 241) presented evidence 1, that the reduction in effective blood volume and changes in blood chemistry after adrenalectomy are similar to those of shock and hemorrhage; 2, that animals in a profound state of shock can be revived by injection of cortical extracts and some purified products; 3, that adrenalectomized animals withstand less trauma and hemorrhage (cf. also 85); 4, that adrenalectomized animals regain their normal resistance if previously protected with large doses of cortical extracts, and 5, that shock cannot be produced by standard methods in normal animals who receive prophylactic doses of cortical hormone. They, therefore, suggested that the adrenal cortical hormones maintain normal capillary tone and permeability. As previously pointed out (p. 99), the evidence that the effects are due to an influence on capillaries is circumstantial. In somewhat similar experiments, Heuer and Andrus (129) also obtained beneficial effects, but only when cortico-adrenal extracts were given early. Perla et al. (194) believed that the resistance of rats to histamine is increased but this apparently occurs also after destruction of the adrenal medulla (137). Karody et al. (141) noted restoration of the histaminase content of the lungs after administration of cortico-adrenal extracts. Selye and his associates (229, 230) developed the concept that the adrenals, interrelated with the thymus and lymphatic tissues, furnish a defense mechanism whereby many tissues resist the deleterious effects of exposure, burns, spinal shock, acute infections, intoxications, etc. It may be theorized that, in some unknown way, additional amounts of appropriate adrenal substances are liberated by the cortex and that tissues gain an additional resistance as a result. If, however, the insult is prolonged or too severe the amount that can be provided

under stress is not adequate; hence cellular dysfunction, presumably including that of capillaries, begins. The fundamental idea seems to be a renovation of a familiar theory advanced some years earlier by Crile. It obviously requires many more facts for its establishment; but it does direct attention to a possible rôle of endocrine factors in resistance to shock. The promptness with which cardiac output and arterial pressures recover after administration of cortical substances to shocked animals is difficult to harmonize with the view that the only effect is to restore capillary tone and permeability, particularly since such restoration has not been demonstrated. The pharmacological actions of these substances on the heart and circulation also require a careful study in connection with such recoveries.

Translocation of potassium. The bulk of experimental work indicates that blood potassium increases under a variety of conditions involving excessive loss of sodium and water from the body, among them hemorrhage, shock due to trauma, intestinal obstruction or fistulas and injection of glucose intraperitoneally, etc. (For reviews see Scudder, 226; Fenn, 81; Harkins, 117.) Since all of these conditions eventuate in circulatory failure, Zwemer and Scudder (269), following a hint by Osterhout, put forward the hypothesis that the release of potassium by cell injury represents the long sought toxic agent in shock. Except in very extensive traumatization of tissues or wholesale destruction of red cells, such increase in potassium could scarcely be an initiating factor. Even in these conditions, its elimination in the urine and bile and its absorption by the liver would probably prevent the rise of blood potassium to toxic levels, much as happens upon ingestion of large doses of potassium salts. Furthermore, Thaler (243) has shown that the progressive increase in blood potassium which follows successive hemorrhages can be reduced again by re-injection of blood or saline, indicating the great mobility of potassium without tissue destruction. Fenn interprets such migration as evidence that intracellular, as well as extracellular, fluids are concerned in attempts to maintain a normal blood volume during hemorrhage.

It is conceivable, however, when the cell machinery begins to break down as a result of prolonged hypotension, that potassium is no longer able to diffuse back into cells and cannot be excreted. Then, the degree of hyperkalemia—or reduction in intracellular potassium—might be a deciding factor as to whether recovery of the circulation is possible or not. This would be in accordance with some observations that the most pronounced rise of serum potassium generally occurs during late

stages of shock (Scudder) and that reduction in blood flow itself increases the concentration of potassium (80), though not as much as was indicated by previous observations of Baetjer (8). While some investigations indicate that blood potassium increases during shock (for references see Scudder), others fail to find a consistent increase or a relationship to the severity of shock (25, 239, 240). Furthermore, elevation of potassium has been reported in other diseases, notably asthma and renal disorders, without evidence of shock symptoms (review, Myers and Muntwyler, 180).

The idea that hyperkalemia is a precipitating factor is not consonant with the fact that the cardiovascular changes which follow injection of potassium and those which occur in shock are strikingly different. Upon injection into normal animals, potassium causes vasoconstriction (62), whereas it is generally admitted that the vessels dilate in terminal stages of shock. There is no evidence that capillary congestion, edema and other signs of shock follow hyperkalemia. Large doses of potassium, administered by vein, affect cardiac rhythm and conduction before contractility and may lead to fibrillation (254, 261). Infusions of isotonic solutions over an hour are less harmful, but show significant changes in the T wave and ST segment develop when plasma concentrations reach 5-10 m.Eq. per liter, and cessation of the heart beat occurs when they reach about 14-16 m.Eq. per liter (254). Similar changes have *not* been reported during the course of shock due to *any* agent. The possibility, of course, exists that conditions are not comparable. Studies of a possible accelerating effect of potassium injected during the course of shock experiments have not been made. After injection of potassium, its intracellular concentration tends to increase, whereas during shock it decreases. Furthermore, serum calcium is reported to rise during shock, and this has some ameliorating effect on the toxicity of potassium (254).

We must conclude that while hyperkalemia has not been proved to be a factor which precipitates circulatory failure, this important complication of shock deserves further study.

Reduction of blood volume to a critical minimum. According to a popular view, serious circulatory failure is precipitated when the effective circulating volume has been reduced to a level at which it can no longer be compensated by vasoconstriction, mobilization of blood from reservoirs and resorption of tissue fluid. With inadequate cardiac output, arterial pressures fall to low levels. Lowered arterial pressure combined with increasing viscosity reduces the pressure transmitted through capillaries into veins, thereby decreasing the primary venopressor force

still further. Obviously, a vicious cycle is started—venous return and cardiac output decrease more, etc.

If such a vicious cycle determined the irreversible state, venous pressure should continue to fall until the end. My observations (257) indicate that, after reaching a lower level, it remains stationary or may even rise somewhat toward the end. Furthermore, large infusions of blood or plasma are of temporary benefit only; the injected blood lodges in the spleen and intestinal vessels and cannot be returned to the heart (personal observations with Werle). Apparently, at a certain stage an adequate circulation cannot be restored by merely filling the system as one does an automobile radiator.

We may conclude that progressive reduction of blood volume *per se* is not the precipitating factor but probably is indirectly responsible for it.

Default of compensatory or emergency mechanisms. Rein (206) has recently summarized investigations from his laboratory which suggest that failure or fatigue of certain emergency mechanisms may constitute the precipitating factor. These mechanisms include: 1. Local chemical agents and axon reflexes which cause constriction or relaxation of minute vessels in accordance with local needs. 2. Extrinsic reflexes from surrounding tissues or from special organs (e.g., in the kidney and mesentery) which exercise a determinate influence on the regions in which they arise. 3. Intrinsic vascular reflexes, mediated essentially by the sinus and aortic nerves, which help to maintain an adjusted action of the cardiac pump, and an adequate aortic pressure so that localized mechanisms can operate. 4. Collateral reflexes which apparently exempt regions that require a better blood flow from participation in the emergency reactions.

We can probably assume that such variable and often conflicting adjustments of peripheral pressures and flow are not a matter of chance but are integrated, to some extent at least, by the central nervous system. Furthermore, the possibility exists that either as a result of bombardment by afferent impulses, e.g., from the site of trauma, or owing to decrease in blood flow to the brain and cord (95, 198), the efficiency of such integration is reduced or abolished altogether during the development of a progressive circulatory imbalance. The conception accords with the fragmentary observations that carotid sinus reflexes are less effective when blood pressures are low (147, 236), that they fatigue easily with repetitive clamping of the carotids (72), and that animals whose blood pressure has been reduced by hemorrhage or trauma withstand less additional hemorrhage or trauma than those whose blood pres-

sure is lowered by vasodilatation (217). Such diminished resistance also follows sympathectomy (93) and previous removal of the spleen (153). However, section of the carotid sinus and aortic nerves still produces a further fall in blood pressure after hemorrhage (185, 236).

If as some experiments indicate (31, 33, 159, 190, 230a, 231, 232), but other investigations do not support (22, 53), afferent impulses play a rôle in production of shock they may do so through disorganization of central integrated control of the circulation. Obviously, this is a problem of neurophysiology as well as one of cardiovascular dynamics, and requires specialized apparatus and training for its study. We cannot hope to solve these problems with equipment restricted to a mercury manometer, an induction coil, and a hammer for pounding muscles.

Vasomotor failure. Extensive reflex arteriolar dilatation may occur as an incipient reaction *during* many catastrophies which lead to shock. These should not be confused with vasomotor failure for it appears to have been amply demonstrated that the vasomotor center remains active and perhaps hyperactive during the development of shock. However, many investigators (50, 77, 178, 199) who reported decreased regional flow during the progress of hemorrhage or shock, found an increased flow⁵ during late stages. Consequently, more careful study of the temporal relation of such changes to the blood pressure may reveal that rapid vasomotor failure constitutes a precipitating mechanism. The sudden circulatory collapse which occasionally occurs under a variety of experimental conditions and unhappily terminates an important experiment is difficult to explain otherwise than by such vasomotor failure. We have seen it occur during dynamic experiments in which venous and initial intraventricular pressure as well as cardiac output remained unimpaired while blood pressure steadily fell. Infusions of saline solution or blood or stimulation of pressor nerves are relatively ineffective in restoring arterial pressures, whereas epinephrine, neosynephrin and related substances increase arterial pressures more than can be attributed to increased cardiac output. Other experimenters have undoubtedly had similar experiences. Ivy (personal communication), for example, informs me that he has observed similar reactions after stimulation of the central vagus under certain conditions, and following insult to the lungs and pleura.

Werle, Cosby and I, recently found that, when extreme hypotension has existed for several hours as a result of regulated bleeding, simultaneous stimulation of both vagus nerves still causes tremendous pressor effects in some dogs, but none in others. Since epinephrine and neosyne-

⁵ At equivalent perfusion pressures.

phrine still cause large elevations of pressure in the latter, we suspect that vasomotor center failure was the precipitating mechanism in these animals. At any event, the time has arrived when it should no longer be considered a sin to give thought to the possible rôle that the vasomotor center may play in determining the lethal character of peripheral circulatory failure.

Myocardial depression. The conception that the myocardium is not affected during shock has become almost an axiom among experimenters. This is doubtless an overstatement and is chiefly based on demonstrations that saline or blood infusions produce, at least temporarily, an increased cardiac output and significant elevations of arterial pressures toward normal. However, the proposition that cardiac filling and expulsion are just as effective in the shocked as in the normal animal at equivalent filling pressures has never been put to an experimental test. In other words, the applicability during shock of laws formulated by Henderson and Starling has not received a thorough study. We have been impressed with the possibility that the myocardium is depressed by three common observations in advanced stages of shock, viz.: 1. In some dogs, venous pressure tends to rise again, suggesting passive congestion. 2. The heart tends to slow after hemorrhage or shock has progressed. 3. Infusion of blood or plasma may raise venous pressures far above normal levels without producing the anticipated increase in cardiac output or blood pressure. 4. When artificial respiration is maintained, progressive myocardial failure definitely terminates any shock experiment.

Myocardial depression may moreover be predicted *a priori* since prolonged hypotension obviously decreases coronary flow significantly. This is compensated to some extent by the diminished work which the left ventricle has to perform, but the work of the right ventricle does not decrease as significantly. Unless, therefore, unknown compensatory reactions occur which augment coronary flow beyond that anticipated from hemodynamic considerations, an anoxic depression of the myocardium seems probable. Therefore, the possibility that depression of the myocardium through impairment of coronary flow or insidious action of possible toxic agents may represent the critical factor for recovery or complete failure of the circulation, deserves further study. That some primary depression of the myocardium exists in some instances seems certain; whether it is of decisive importance in the causation of irreversible circulatory failure still remains a question.

The problem of aortic adaptation. In discussing the possible increase

in the capacity of the vascular system during shock only the dominant changes in the smaller vessels of the circuit are generally considered. That the capacity and elasticity of the aorta and its branches may likewise be altered during shock has until recently seemed highly improbable. Reports can be found in the literature which indicate that the size of the aorta and its immediate branches (223) may not be adjusted entirely passively by the internal pressures (for review see Bazett, 19). In view of the paucity of muscular elements demonstrable histologically, and their questionable arrangement for effective action, the writer has been among those who regarded such reports as based on concealed technical errors. Quite unexpectedly, however, Wegria and I (263) found during temporary hypertension produced by reflexes and epinephrine, that the aorta, after a temporary (passive) expansion, progressively decreased in size.

Preliminary experiments reported by Werle and Cosby (251) showed that some dogs, after maintenance of a low arterial pressure by repeated hemorrhage, failed to recover when the blood withdrawn was all reinfused. A study of the forms of the optical pressure pulses recorded after such reinfusion showed significant changes. As in previous experiments on shock reported by the writer (257), these changes might be due to a greatly reduced peripheral resistance. They could, however, equally well be attributed to a different mode and volume of ejection or to a relaxed state of the aorta. The remote possibility that failure of mechanisms which adapt the size and elasticity to changing volumes and pressures of blood may be a decisive factor in circulatory failure needs to be investigated.

Summary of precipitating factors. Our own impressions and those gleaned from a review of pertinent literature on shock are that while venous return is reduced relatively early in shock and represents the chief factor in its continuance, such a decrease alone does not suffice to create the irreversible circulatory failure characteristic of deep shock. Consequently, the suggestion is ventured that some as yet unidentified precipitatory mechanism or several of these in association exists. A number of possible factors—the adrenal cortex and its hormones, translocation of potassium, default of emergency reflex controls, vasomotor failure, myocardial depression and lack of aortic adaptation—are suggested for further study in this connection.

THE SEQUENCE OF DYNAMIC EVENTS DURING HEMORRHAGE AND SHOCK. During the past year (1940–41), Doctors Werle, Cosby and I performed miscellaneous experiments on hemorrhage, designed to cast further light

on moot dynamic problems arising from a coincident re-survey of recent literature for this review. As a result, my conceptions of the sequence of events in hemorrhage (255) and shock (257) have been revised somewhat, as follows: The similarity of changes (quantitative and qualitative) in the central arterial pressure pulses during hemorrhage and shock indicate that the dynamic sequence is the same in both; except that, in traumatic shock, vasodilatation may be a temporary prelude. This is, in fact, comparable to primary shock; it is caused by direct actions or local reflexes in intestinal exposure and manipulation and, reflexly, in trauma accompanied by other intensive nervous reactions. The transient character of this reaction precludes its detection in connection with time-consuming gasometric measurements of cardiac output, and it is, therefore, consistently missed in such studies. It is still questionable whether these preliminary changes have any bearing on the subsequent secondary shock that develops.

Owing to the operation of compensatory mechanisms, the circulating volume may be slowly decreased by a significant amount (ca. 35 per cent) before arterial pressure declines; in fact, it may elevate. This well-known fact has been conveniently explained by acceleration of the heart and increased total peripheral resistance, due to fairly generalized vasoconstriction. Evidence now available makes it more probable that blood pressure is sustained chiefly because cardiac output is not decreased at once. In order to accomplish this a normal venous return must be maintained. My present conception is that vasoconstriction is not universal and acts not so much by increasing total peripheral resistance—which is doubtful—as by driving blood from certain blood reservoirs—spleen, skin and liver—into the large veins. Whether the veins contract in addition, as some (39, 101, 127) have claimed, I am not prepared to say. Vasoconstriction is also helpful in reducing capillary pressures in selective areas, thereby favoring the resorption of tissue fluid, which according to Adolph (1) is practically complete within 22 minutes after a single large hemorrhage. This constitutes a second line of defense in maintaining a normal venous return. During this early stage of hemorrhage there is no determinable increase in the oxygen utilization coefficient nor impairment of blood flow (99), hence *initial* deterioration of capillary tone through vasoconstriction seems to be a figment of the imagination. Capillary damage is more probably a consequence than a cause of arterial hypotension.

The second stage, generally inaugurated by a moderate decline of arterial blood pressure and striking changes in the contour of the pres-

sure pulses begins when compensatory mechanisms are no longer able to maintain venous return; consequently, cardiac output decreases. Whether additional vasoconstriction at this stage can compensate by effecting an increased total peripheral resistance requires further study; this probably varies in different animals. In accordance with all evidence, the progressive decline in blood pressure to lower levels is almost wholly due to a progressively decreasing cardiac output. During these stages, reinfusion of blood or plasma leads to complete recovery. Following this, either of two events supervenes: The circulatory failure may continue fairly steadily on its downward course or, after blood pressure has been stabilized for several hours at levels ranging from 78 to 50 mm. Hg, it drops rather abruptly, and death occurs with certainty within one-half to one hour.

In the latter type, precipitating mechanisms are almost certainly concerned. The abrupt intensification of the downward course must be differentiated from one due to cessation of respiration, an occasional accident in anesthetized animals. While it occurs during the period of decreasing respiratory rate and depth, we have so far been unable to link the sudden failure to a possible arterial anoxemia or to default of a respiratory pressor influence on venous return, but this deserves further study.

Two precipitating causes for the circulatory collapse which follows seem to operate, failure of the vasomotor center and failure of the heart; but the operation of other mechanisms cannot be excluded. After the irreversible failure has begun, transfusions of blood or plasma may restore cardiac output, but blood pressures and pressure pulses are never restored to normal and the improvement is transient. Within another hour or less, progressive failure rapidly leads to a similar mode of death. Inspection of viscera during the course of such temporary recovery and decline, or at autopsy, reveals that much of the transfused blood accumulates in the spleen and mucosa of the small intestines. As engorgement of the mucosal vessels increases, some rupture and cause a bloody intestinal fluid. The stomach and colon are not congested but generally remain pale. Such observations clearly indicate that something happens in the peripheral circulation during late stages of hemorrhage which leads to congestion and rupture of capillaries. The evidence available in the literature reviewed does not as yet permit expression of a final opinion as to how this is brought about.

In conclusion, if in these discussions the author has appeared unnecessarily disputatious in accepting generally favored notions, let it be understood that counter-emphasis in the interpretation of known facts

is sometimes made designedly in the hope that others may be stimulated to produce more crucial evidence or more cogent arguments for their beliefs.

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EDITORIAL NOTICE

War duties of primary importance are delaying many authors in the completion of their contributions. Therefore it is probable that subsequent issues of *Physiological Reviews* will be reduced in size. It is hoped that compensation for this situation can be made after the emergency.



PHYSIOLOGICAL REVIEWS

VOL. 22

APRIL, 1942

No. 2

BLOOD-BRAIN BARRIER

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In view of its ramifications in many branches of medicine (physiology, bacteriology, immunology and pharmacology), the problem of the blood-brain barrier is of more general interest than this term seems to indicate. A critical review of the widely scattered literature, therefore, may be of substantial help to those who are interested in the various aspects of the subject. This paper deals with the experimental evidence for the existence of this barrier, its localization, permeability to aniline dyes, toxins, viruses, antibodies and drugs, and with the physico-chemical factors controlling this permeability. The significance of these experiments for problems of pathogenesis and the physiological problem of capillary permeability will be discussed. It will be seen that on this broader experimental basis some fundamental questions can be definitively answered which were not decided by the rather one-sided consideration of experiments with aniline dyes.

It may be emphasized at the outset that this paper deals exclusively with the distribution of substances between blood and C-N-S. As will be shown, distribution between blood and cerebrospinal fluid (C-S-F) is an entirely different problem and remains outside the scope of this review.

I. *Fundamental facts.* The existence of a barrier between blood and brain was first suggested by the observation that certain substances, either colored or toxic, when injected into the vascular system, fail to produce any changes in the C-N-S, while they have marked effects after intracerebral, intraventricular or intrathecal injection. Such experiments have been reported with bile by Biedl and Kraus, with diphtheria toxin by Roux and Borrel, with potassium ferrocyanide by Lewandowsky and with trypan blue by Goldmann.

In another series of experiments it was shown that certain pathogenic agents reach the C-N-S exclusively by neural pathways. This was claimed for tetanus toxin by Meyer and Ransom and for the majority of neurotropic viruses by a large number of investigators. To what extent these fundamental observations actually prove the existence of a barrier between blood and C-N-S will be discussed in the following sections. Further experimental evidence pertaining to the subject will be presented.

II. *Older theories on the exchange of substances between blood and C-N-S.* v Moakow and Stern considered the C-S-F to be the nutrient fluid of the C-N-S. They, consequently, assumed that the exchange of substances between blood and C-N-S takes place exclusively through the C-S-F. If this were true, the permeability of the blood-brain barrier could be tested by an examination of the C-S-F. Actually a large number of clinical investigations have been carried

out on this basis. Although Spatz, (135, 136, 137) Riser, (21, 22, 23, 126) Walter and Morgenstern and Birjukow have raised serious objections to this theory it has been widely accepted and still finds its place in some otherwise excellent textbooks.

On the basis of general physiological considerations and experimental facts, however, this theory appears hardly acceptable. It is utterly unlikely that in an organ of the vital importance of the brain the capillaries should lack their chief uses, namely, mediation of the exchange between blood and tissue and adaptation of blood supply to functional needs.

In addition, Wesselkin, Friedemann and Elkeles (62) and Schmid invalidated the experimental foundation of this theory. Stern and Gautier claimed to have demonstrated a strict parallelism between the appearance of intravenously injected substances in the C-N-S and the C-S-F. The above mentioned authors, however, found independently that intravenously injected basic aniline dyes stained the brain intensely without appearing in the C-S-F. According to Friedemann and Elkeles this fluid is not colored even if the lateral ventricles and the cisterna magna are punctured at the moment when the exposed cortex becomes colored. Moreover, any transport of these dyes through the C-S-F could be ruled out by the observation that the grey matter was colored throughout the entire brain.

Experiments with acid dyes are of equal importance. As will be shown in the following section their intravenous injection leaves the brain entirely uncolored although, according to Wittgenstein and Krebs (161a) they appear in the C-S-F. This shows that the rate of absorption from the C-S-F into the blood is higher than that of their diffusion from the blood into the C-S-F. All these observations lead to the conclusion that the C-S-F has no appreciable part in the exchange of substances between blood and brain and that, consequently, as in other organs, this exchange takes place directly between blood and tissue.

It should be emphasized that the localization of the blood-brain barrier in the choroid plexus would be unjustified even if the exchange of substances between blood and brain took place solely by way of the C-S-F. It is obvious that in this case the cerebral capillaries would be impermeable to all substances circulating in the blood.

An entirely different conception of the exchange of substances between blood and tissues was advocated by Krogh and Ehrlich. Both authors deny the existence of any selective capillary permeability. According to Ehrlich (41) this exchange is controlled by chemical affinities. A similar opinion was recently voiced by King in a discussion of Goldmann's experiments with trypan blue. In the following sections it will be shown that the inability of certain substances to reach the C-N-S from the blood is actually due to a selective permeability of the cerebral capillaries. At the same time, with the aid of various methods, the permeability of these capillaries to aniline dyes, toxins, viruses, antibodies and drugs will be investigated.

III. *The permeability of the capillaries of the C-N-S to aniline dyes.* In 1887

Ehrlich (42) reported that subcutaneously injected acid aniline dyes (with very few exceptions) failed to stain the brain whereas a considerable number of basic aniline dyes gave positive results. However, the majority of them produced no staining. Mandelstamm and Friedemann (57, 58) found independently that this latter result holds true only if the dyes are injected subcutaneously. After intravenous injection almost all basic dyes stain the brain definitely. Ehrlich's negative results with acid dyes were confirmed by Goldmann, Spatz (138) and Friedemann (58).

The importance of these results for the theory of the blood-brain barrier is obvious. Concerning their interpretation, however, opinions are still divided. Only recently King has suggested that the negative results obtained with trypan blue are due to the fact that this dye has no affinity for any tissue elements in the C-N-S. In his opinion, trypan blue has a specific affinity for connective tissue which is absent from most of the brain.

Goldmann's well known second experiment was designed to rule out this explanation. It was shown that when introduced into the subarachnoid space, the dye stained the brain intensely. The experiment of Goldmann, however, is open to some serious objections. In the first place, it must be kept in mind that the volume of the C-S-F is only $\frac{1}{10-20}$ of that of the blood plasma. For this reason alone, when injected into the subarachnoid space, trypan blue should be 10 to 20 times more effective than after its injection into the vascular system. Actually Lewandowsky found that strychnine, although it certainly passes the capillaries of the C-N-S, was ten times more toxic by the intrathecal than the intravenous route. Moreover, according to Friedemann (56) and Bennhold, aniline dyes combine with the proteins of the blood plasma whereby their diffusibility is considerably diminished. Consequently the results of the subarachnoid and intravenous injections are not comparable.

To avoid this difficulty Friedemann (58) replaced the subarachnoid injection by an in vitro test. Small bits of brain were put into various dilutions of the individual dyes in blood serum and the minimal staining concentration (C_v) was determined. The minimal staining amounts of the intravenously injected dyes divided by the plasma volume give the minimal staining concentration in the blood plasma (C_B). Theoretically C_v should be equal to C_B if the cerebral capillaries were perfectly permeable. Table 1 shows that within the limits of experimental error this actually holds true for basic aniline dyes. As may be seen from table 2 the results are very different with acid dyes.

Table 2 shows that for acid dyes the values of C_B are high multiples of those for C_v .¹ This result would be inexplicable were the difficulty of acid dyes to reach the brain from the blood due only to their lack of affinity for nerve tissue. On the contrary, it shows that the capillaries of the C-N-S are either entirely

¹ According to Spatz (138) the faint staining obtained with large doses of intravenously injected trypan blue is a result of the dye content of the cerebral vessels. The real value of $\frac{C_B}{C_v}$ for trypan blue, therefore, would be considerably larger than that recorded in table 2.

impermeable or very little permeable to acid aniline dyes. These experiments with basic and acid dyes, therefore, are conclusive proof for the selective permeability of the capillaries of the C-N-S.²

Goldmann believed to have proven by his experiment that trypan blue has a definite affinity for nerve tissue but is prevented from reaching the brain by what is now called the blood-brain barrier. This explanation is probably not correct. Spatz (138) has shown that after a single subarachnoid injection of the dye the ganglion- and glia cells are not stained within the blue zone at the lower surface of the brain. Trypan blue reaches the nerve tissue merely by way of diffusion. These observations show clearly that chemical affinity is no prerequisite for

TABLE 1
The values of C_B , C_V and $\frac{C_B}{C_V}$ for some basic aniline dyes

| DYES | MINIMAL STAIN- ING AMOUNT (INTRAVENOUS) | C_B | C_V | $\frac{C_B}{C_V}$ |
|---------------------|---|----------|----------|-------------------|
| | <i>mgm.</i> | | | |
| Bismark brown..... | 2.5 | 1: 4,000 | 1: 2,500 | 1.6 |
| Nile blue..... | 1.0 | 1:10,000 | 1:10,000 | 1.0 |
| Chrysoidin..... | 1.0 | 1:10,000 | 1:10,000 | 1.0 |
| Methylene blue..... | 1.25 | 1: 8,000 | 1:10,000 | 0.8 |
| Pyronin..... | 2.0 | 1: 2,500 | 1: 5,000 | 0.5 |

TABLE 2
The values of C_B , C_V and $\frac{C_B}{C_V}$ for some acid dyes

| DYES | MINIMAL STAIN- ING AMOUNTS (INTRAVENOUS) | C_B | C_V | $\frac{C_B}{C_V}$ |
|-----------------------|--|--------|---------|-------------------|
| | <i>mgm.</i> | | | |
| Uranin..... | >80 | >1:125 | 1:2,000 | >16:1 |
| Eosin..... | >40 | >1:250 | 1:2,000 | >8:1 |
| Trypan blue..... | 40 | 1:250 | 1:1,000 | 4:1 |
| Congo red..... | 80 | 1:125 | 1:2,000 | 16:1 |
| Indigodisulphate..... | 20 | 1:500 | 1:5,000 | 10:1 |

macroscopic staining of tissues, provided the dye is able to reach them. On the other hand, despite their affinity for nerve tissue pathogenic agents may be barred from reaching the C-N-S by the capillary endothelium. This will be demonstrated by the following experiments with toxins and neurotropic viruses.

IV. *The permeability of the capillaries of the C-N-S to toxins.* A conflicting literature illustrates the difficulties in establishing the central origin of toxic symptoms on the basis of clinical observations and physiological investigations.

² More comprehensive quantitative data concerning the staining of the brain by intravenously injected basic and acid aniline dyes have been reported by Friedemann (58) in a previous paper.

In addition, a specific action of a toxin on the C-N-S does not necessarily mean that it reaches it directly by way of the circulation. On the other hand, a toxin may fail to act on the C-N-S because it lacks any affinity for nerve tissue. The investigation of the permeability of the capillaries of the C-N-S, therefore, requires special methods which will be described in the following sections.

A. *Tetanus toxin*. In a series of ingenious experiments Meyer and Ransom claimed to have proven that tetanus toxin reaches the C-N-S exclusively by neural pathways. Recently, however, part of the experimental evidence on which this theory was based has been severely criticized by Horster and Whitman, Doerr, Seidenberg and Magrassi and Abel and his co-workers. Abel and more recently Doerr (29) arrived at the conclusion that, like other toxins, tetanus toxin reaches the C-N-S directly by way of the circulation and that the symptom of local tetanus is due to a direct action of the toxin on the muscle. Friedemann, Zuger and Hollander (70, 71) and Friedemann, Hollander and Tarlov have reinvestigated the problem with the aid of new methods. Concerning experimental details the reader may be referred to the original papers. The following results were obtained.

1. Intramuscularly injected tetanus toxin reaches the C-N-S even if any transport through the circulation is excluded by a large excess of circulating antitoxin.
2. Tetanus toxin reaches the C-N-S by way of the motor nerves.
3. Local tetanus is of central origin.

The third point is of particular importance for our problem. It is obvious that after exclusion of the peripheral origin of local tetanus this symptom could not be explained if tetanus toxin reached the C-N-S by way of the circulation. The capillaries of the C-N-S, therefore, are impermeable to tetanus toxin.

B. *Diphtheria toxin*. The question of the permeability of the cerebral capillaries to diphtheria toxin was first investigated by Bieling and Gottschalk. They injected large doses of toxin intravenously and determined the toxin contents of various organs by Rocmer's method. No toxin was found in the brain. The almost complete absence of toxin from the liver, however, casts some doubt on the reliability of this procedure. We have reinvestigated this problem in a series of as yet unpublished experiments. They were based on an old observation of Roux and Borrel recently confirmed by Doerr and Kon and Bieling and Oelbrieh. As mentioned before, the rat is almost immune to diphtheria toxin given by the ordinary routes while highly susceptible to its intracerebral injection. Roux and Borrel explained this observation on the assumption that in the rat the toxin is fixed by other organs and, thereby, deviated from the brain. Using Rocmer's method for identifying the toxin in the blood, it was found that the toxin does not disappear more rapidly from the circulation of the rat than from that of the rabbit or guinea pig, which are highly susceptible to injection by ordinary routes. One and a half hours after the intravenous injection of 1 cc. of toxin, the blood of the rat still contained 80 intracerebral lethal rat doses. Dr. H. Zimmerman of New Haven examined the brains of our rats histologically. Even after the intraperitoneal or intravenous injection of 0.5 to 1 cc. of toxin no lesions were present, whereas the intracerebral injection of a single lethal dose

(0.005 cc.) was followed by marked pathological findings. All these experiments, taken together, leave little doubt that in the rat the capillaries of the C-N-S are impermeable to diphtheria toxin.

The question is more complicated in the case of the rabbit and the guinea pig. The following facts, however, seem to indicate that the impermeability of the cerebral capillaries to diphtheria toxin also holds true for the rabbit. Friedemann, Hollander and Tarlov have shown that in order to prevent local tetanus after intramuscular injection of the toxin, antitoxin is far more effective by the intraventricular than the intravenous route. Friedemann and Elkeles (59), however, in experiments on rabbits, found that the protecting dose of antitoxin against intravenously injected diphtheria toxin was exactly the same by the intraventricular and the intravenous routes. In connection with the tetanus experiment this result tends to show that after intravenous injection diphtheria toxin does not act on the C-N-S of the rabbit. Since, according to Caporali and Friedemann and Elkeles (59), diphtheria toxin is from 20 to 40 times more toxic by the intracerebral than the intravenous route, its affinity for nerve tissue is beyond doubt and its inability to reach the C-N-S from the circulation can be explained only by its failure to pass the capillaries of the C-N-S.

In view of these results the theory of Romberg, Paessler, Bruhn and Mueller that circulatory failure in the early stages of diphtheria is due to the action of the toxin on the vasomotor center in the medulla is no longer tenable.

C. Botulinus toxin. Physiological investigations of Dickson and Shevsky and Bishop and Bronfenbrenner have shown that botulinus toxin acts on the peripheral nerves. In conformity with these findings, Friedemann and Elkeles (65) arrived at the conclusion that the toxin does not reach the C-N-S from the circulation. One hour after the intravenous injection of 0.1 cc. of botulinus toxin into mice of 20 grams, 1 cc. of blood contained 1,000 intraperitoneal M.L.D. whereas the brain was found to be free from toxin. As a matter of fact Forssman has shown in interesting immunological experiments that botulinus toxin has no affinity for the C-N-S. The aforementioned experiments with trypan blue, however, have shown that, despite a lack of affinity, a colloidal substance may diffuse into nerve tissue unless it is prevented from doing so by an impermeable barrier. We, therefore, are led to the conclusion that the capillaries of the C-N-S are impermeable to botulinus toxin.

D. Dysentery toxin. After intravenous injection the toxin of the Shiga dysentery bacillus produces paresis in rabbits. The patchy and hemorrhagic character of the histological lesions in the C-N-S (Doerr and Seidenberg, 34), however, indicates that the capillary system is damaged by the toxin. It is impossible, therefore, at the time being to determine the permeability of the normal capillaries of the C-N-S to this toxin.

E. Cobra venom, lamb dysentery and staphylococcus toxins. These three toxins are discussed together because, contrary to the first four toxins, they act rapidly without any noticeable incubation period. This makes possible the application of two methods devised by Friedemann and Elkeles.

In the first method the brain of one rabbit, the recipient, is perfused by the

blood of another rabbit, the donor. After ligation of the vertebral arteries of the recipient both carotid arteries and jugular veins of the two animals are anastomosed. The toxins are injected either into the circulation of the donor or into the femoral vein of the recipient. The details of the method and some of its applications have been described by Friedemann and Elkeles (60, 61).

The second method is based on the observation of Friedemann and Elkeles (63) that adrenaline causes a 5- to 10-fold increase in the permeability of the cerebral capillaries to those substances to which they are already permeable (ethylurethane, ethylalcohol, paraldehyde, strychnine and alizarinblue-S). The impermeability to other substances (arsphenamine, trypanblue) remains unaltered. Consequently, adrenaline enhances the toxicity of those toxins which pass the capillaries of the C-N-S while it does not affect those which fail to do so. This method will be spoken of as the method of the auxoneurotropic effect.

Artificial perfusion of the brain was not applicable to cobra venom. The well known action of this toxin on the arterioles (Elliot) interrupted the perfusion at once. Adrenaline, however, reduced the rabbit intravenous lethal dose from 0.5 mgm. to 0.025 to 0.05 mgm. The capillaries of the C-N-S, therefore, are permeable to cobra venom (65).

Adrenaline also increased the toxicity of intravenously injected lamb-dysentery toxin³ 4 times. In this case artificial perfusion also gave significant results. When the toxin was injected into the femoral vein of the recipient the cerebral reflexes (corneal, lip and tongue) disappeared at once. Obviously, it had reached the brain through anastomoses between the vertebral and spinal arteries. Both methods, therefore, led to the conclusion that the capillaries of the C-N-S are permeable to lamb-dysentery toxin (65).

Contrary to the results obtained with cobra venom and lamb-dysentery toxin Friedemann (58) found that adrenaline had no influence on the toxicity of intravenously injected staphylococcic toxin. The drug had even a slight protecting effect. It must be concluded, therefore, that staphylococcus toxin does not reach the C-N-S from the circulation. On the other hand the toxin was found to be 10 times more toxic by the intracerebral than the intravenous route. These results clearly indicate that the capillaries of the C-N-S are impermeable to staphylococcus toxin. On the basis of pharmacological studies Burnet and Kellaway arrived at the same conclusion.

V. *The permeability of the capillaries of the C-N-S to neurotropic viruses.* It is now generally accepted that, if injected into tissues, the majority of neurotropic viruses reach the C-N-S exclusively by neural pathways. In the opinion of Hurst this indicates that the blood-brain barrier is impermeable to these viruses. His views, however, are not accepted by Doerr (28, 29). Actually the conclusions of Hurst would be convincing only if it could be shown that after peripheral injection these viruses circulate, at least for some time, in the blood stream. This certainly does not hold true for the strictly neurotropic viruses, namely, the viruses of poliomyelitis, rabies, and Borna disease. It is doubtful in the case of

³ The lamb-dysentery bacillus produces diarrhea in newborn lamb. It is an anaerobic organism of the perfringens group.

herpes simplex. The permeability of the capillaries to all these viruses, therefore, can be tested only by their intravenous injection. From this point of view the entire literature on neurotropic viruses has been reassessed. The interesting material will be published elsewhere. In view of the limited space I must confine myself to the results. The evidence is conclusive or highly suggestive that the viruses of poliomyelitis, rabies, Borna disease, herpes simplex, louping ill, pseudorabies, St. Louis encephalitis, vesicular stomatitis, the neurotropic strain of yellow fever and equine-encephalo myelitis in mice and old guinea pigs are unable to pass the capillaries of the C-N-S. In the case of virus B, the existing experimental evidence is inconclusive.

Some neurotropic viruses pass the capillaries of the C-N-S but not in a physical sense. They infect the capillary endothelium and thus grow through the capillary membrane. This has been shown to hold true for equine encephalomyelitis in young guinea pigs and canine distemper.

The virus of fowl plague assumes a peculiar position. This is a pantropic virus but Doerr and Seidenberg (33), working with a highly virulent strain in chickens, have shown that the brain becomes infected early in the disease. It is very likely, therefore, that this virus passes the capillaries of the C-N-S of chickens. Other experiments of Doerr and Seidenberg (32, 35a) have shown that the same holds true for guinea pigs and mice.

Some observations seem to indicate that the ability of viruses to pass the capillaries of the C-N-S is in some way connected with their virulence. Working with a less virulent strain Lagrange arrived at the conclusion that the virus of fowl plague is unable to pass the capillaries of the C-N-S in the chicken. It has also been found that these capillaries may be permeable to a virus in one species, while impermeable in another. Experiments of Kleine and Moellers seem to indicate that in adult geese the blood brain barrier is impermeable to fowl plague virus. In adult mice the capillaries of the C-N-S are impermeable to the neurotropic strain of yellow fever virus. But they are apparently permeable in young mice (Theiler) and hedgehogs (Findlay and Clarke).

Summarizing we may conclude that in the ordinary laboratory animals and in a physical sense the capillaries of the C-N-S are probably impermeable to all neurotropic viruses if we exclude the virus of fowl plague because of its pantropic character.

VI. *The permeability of the capillaries of the C-N-S to antibodies.* According to the literature the majority of investigators seem to believe that the blood-brain barrier is impermeable to antibodies. This belief is mainly based on the following facts:

1. The unsatisfactory results of serum therapy in tetanus and poliomyelitis.
2. The absence of antibodies from the C-S-F in highly immunized animals.
3. The experiments of Roux and Borrel which showed that rabbits actively or passively immunized against tetanus toxin were not protected against the intracerebral injection of a single lethal dose of the toxin.

Freund, Weichsel and Salfeld, and Abel and Chalian, however, assume that the capillaries of the C-N-S are permeable to antibodies.

Since we have seen that the exchange between blood and C-N-S takes place directly through the capillaries, the second argument apparently is no longer tenable. The experiments of Roux and Borrel, however, need careful consideration. Friedemann, Zuger and Hollander (68, 69), therefore, have submitted them to an experimental analysis. These investigations started from the observations of Descombey and of Mutermilch and Salamon that guinea pigs actively or passively immunized against tetanus toxin were protected against the intracerebral injection of as many as 20 lethal doses of the toxin. Their explanation was that the blood-brain barrier is permeable to antitoxins in the guinea pig but impermeable in the rabbit. Experiments of Friedemann, Zuger and Hollander (68) with diphtheria toxin and antitoxin, however, showed definitely that this explanation cannot be accepted. It was found that in both animals 10 intracerebral lethal doses of diphtheria toxin were neutralized by exactly the same concentration of antitoxin in the blood. This discrepancy between the tetanus and diphtheria experiments can be explained in the following manner. The intracerebral lethal dose of diphtheria toxin is almost exactly the same for the rabbit and the guinea pig. The intracerebral lethal dose of tetanus toxin, however, is approximately 100 times greater for the rabbit than for the guinea pig. When equal absolute amounts of tetanus toxin were given intracerebrally to both species, both were protected by the same concentration of antitoxin in the blood. The difficulty in protecting rabbits against intracerebrally injected tetanus, therefore, is not due to the impermeability of the blood-brain barrier to antibodies but to the high combining power of the test dose of toxin in the rabbit. Since in the guinea pig 0.001 cc. of antitoxin protected against 10 lethal doses of the toxin it becomes apparent that both in the rabbit and the guinea pig the capillaries of the C-N-S are definitely permeable to antitoxins.

With the aid of skin test experiments Friedemann, Zuger and Hollander (66, 67) developed a new method by which the distribution of antitoxin between blood and tissues can be determined quantitatively. It was found that the antitoxin concentration in the intercellular fluid is approximately 1/20 of that in the blood plasma. The method is less accurate for the brain. It may be gauged, however, that the coefficient of distribution of antitoxins between blood and brain is somewhere between 1/20 and 1/60. Despite the permeability of the capillaries of the C-N-S to antibodies their intrathecal application, therefore, appears justified. As mentioned before, tetanus antitoxin is 80 times more potent in preventing local tetanus by the intraventricular than the intravenous route.

VII. *The permeability of the capillaries to proteins.* The experiments with antibodies are of interest in connection with the much disputed question of the permeability of the capillaries to proteins and the protein contents of tissue fluids. While Starling, Krogh, Landis and Peters consider the latter as low, Drinker and his associates believe that they approach that of lymph. Thus far, the protein content of the tissue fluid has been estimated only indirectly from that of the lymph or pathological exudates. As shown in our original paper

our method determines the antibody concentration in the capillary filtrate itself. There is every reason for believing that the distribution of antibodies between blood and plasma runs parallel to the distribution of globulins. Since the albumin/globulin ratio of the tissue fluid is probably a little higher than that of the blood plasma the total protein content of the tissue fluid may also be higher than that calculated from its antibody content. It may amount to 5 or 10 per cent of the protein content of the blood plasma, a figure which fits in satisfactorily with the results of Landis obtained with an entirely different method.

It should be emphasized, however, that our figure holds true only for the skin. In the tissue fluid of the liver the protein content is certainly much higher and in the C-S-F and the aqueous humor it is considerably lower. As mentioned before, the protein content of the tissue fluid in the brain is probably lower than in the skin.

VIII. *The permeability of the capillaries of the C-N-S to drugs.* Thus far no systematic pharmacological studies on the blood-brain barrier have been reported. Until recently it has been taken for granted that substances which act on the C-N-S are able to pass its capillaries. The investigations of Heymans, however, have cast some doubt on the validity of this assumption. He has shown that CO₂, cyanides, nicotine, lobeline, sulfides and acetylcholin influence the respiratory center only indirectly via the carotid sinus.

More reliance may be placed on the identification of drugs in C-N-S. Positive results were obtained with a considerable number of alkaloids and with the sulfonamides (Riser and Valdigné). It may be safely assumed, therefore, that these drugs pass the capillaries of the C-N-S.

On the contrary, McIntosh and Fildes, Rudolf and Bulm, Underhill and Dimick, Kolls and Youmans, Voegtlin and others found that these capillaries are impermeable to nearsphenamine. After intravenous injection of this drug no arsenic or only traces of it were found in the C-N-S. Wallace and his associates found that in other organs the coefficient of the distribution of bromides, iodides and thiocyanates was the same as that of chlorides. Their content in the C-N-S, however, was much lower. The capillaries of this organ, therefore, are relatively impermeable to these drugs. The question of the permeability of the cerebral capillaries to magnesium salts is puzzling. Despite their narcotic action they cannot be identified in the C-N-S (Gensler, Mansfeld and Bosany).

As mentioned before, Lewandowsky found with the aid of a different method that the capillaries of the C-N-S are impermeable to sodiumferrocyanide. Using the same method, Friedemann and Elkeles (65) arrived at the same conclusion concerning Bayer 205 (Germanin). This trypanocidal drug is entirely atoxic by the intravenous route. Injected into the subarachnoid space it was found to be extremely toxic.

IX. *The relation between the ability of substances to pass the capillaries of the C-N-S and their electrical charge.* The investigations reported in the preceding sections have shown that the capillaries of the C-N-S are permeable to basic aniline dyes, cobra venom, lamb dysentery toxin, fowl plague virus, antibodies

and a great variety of drugs. They are impermeable to acid dyes, tetanus, diphtheria, botulinus and staphylococcus toxins, to most if not all neurotropic viruses (in a physical sense), potassium ferrocyanide, arsphenamine, Bayer 205 and relatively impermeable to iodides, bromides and thiocyanates. The striking differences within the individual groups obviously call for an investigation of the physico-chemical factors controlling the permeability of the capillaries of the C-N-S.

As far as non polar substances are concerned there is little doubt that lipid-solubility or surface activity play an important rôle. The following considerations deal only with polar substances. It is a striking feature of our results that molecular size is apparently of little significance. The capillaries of the C-N-S are permeable to the colloidal antibodies, cobra venom, lamb dysentery toxin and even to the corpuscular fowl plague virus while they are impervious to highly diffusible acid dyes. Among basic dyes, the colloidal night blue and victoria blue pass the cerebral capillaries readily.

The clear cut difference between basic and acid dyes points to the importance of the electric charge. Although it will be shown in section XI that this concept is open to some theoretical objections, it was the lead in our further investigations. In the following paragraphs the electrical properties of toxins, viruses, antibodies and some microorganisms will be considered in connection with their ability to pass the capillaries of the C-N-S.

1. *Cataphoresis experiments with toxins.* As may be seen from table 3, Friedemann and Elkeles (65) found, in conformity with our hypothesis, that the ability of toxins to pass the cerebral capillaries is closely related to their electrical charge.

Table 3 shows that the negatively charged toxins (tetanus, diphtheria, botulinus and staphylococcus) do not pass the cerebral capillaries while positively charged cobra venom does. The result obtained with lamb dysentery toxin shows that substances which are isoelectric at the pH of the blood can pass the cerebral capillaries. Unfortunately the number of true toxins is limited. Nevertheless, the agreement between theoretical expectation and experimental facts is too striking to be dismissed as a mere coincidence.

2. *The electrical charge of viruses.* In table 4 are recorded the available data concerning the electrical charge of neurotropic viruses.

As may be seen from table 4 the negative electrical charge of these viruses is in conformity with their inability to pass the capillaries of the C-N-S. It is doubtful, however, whether this is a relationship of cause and effect. Obviously, the factor of size would explain the results as well. It must be remembered, however, that the size of the elementary bodies of some viruses does not exceed that of large protein molecules. Moreover, the cerebral capillaries are permeable to fowl plague virus. The electrical charge of this virus is of the greatest importance for our problem. According to Lépine (97) it is negatively charged over a range of pH 6.2-8.2. The paper, however, gives no information concerning the virulence of the strain used. Dr. A. Todd, of the National Institute for Medical Research, London, (personal communication) carried out

cataphoresis experiments with the highly virulent strain of fowl plague virus used by Doerr and Seidenberg. At the pH of the blood the virus was found to go neither to the cathode nor the anode. The peculiar electrical charge of fowl plague virus is indicated also by investigations of Doerr and Gold who reported that it is much more readily absorbed by the electronegative charcoal than other viruses and bacteriophages. This fits in with its ability to pass the cerebral capillaries. Further investigations along these lines with various strains of fowl plague virus are clearly indicated.

TABLE 3
Cataphoresis experiments with toxins

| TOXINS | pH 8.2 | pH 7.4 | pH 6.2 |
|---------------------|--------|--------|---------|
| Diphtheria..... | A | A | A |
| Botulinus..... | A | A | A |
| Tetanus..... | A | A | A and C |
| Staphylococcus..... | A | A | A and C |
| Lamb dysentery..... | A | O | C |
| Cobra venom..... | C | C | C |

A = toxin goes to the anode; C = toxin goes to the cathode; O = no migration.

TABLE 4
Cataphoresis experiments with neurotropic viruses

| VIRUS | pH | CHARGE | AUTHOR |
|---|-----------|----------|--|
| Louping ill..... | 7.3 | Negative | Lépine (107) |
| Borna disease..... | 6.6-7.4 | Negative | Nicolau and Kopacowska (129) |
| Yellow fever..... | 5.2-7.0 | Negative | Hindle and Findlay |
| Herpes simplex..... | 5.3-7.8 | Negative | Nicolau and Kopacowska (126, 127) |
| Rabies..... | 5.8-7.4 | Negative | Nicolau and Kopacowska (128) |
| Poliomyelitis..... | 6.9-8.0 | Negative | Lévaditi and Lépine |
| Equine encephalomyelitis (Eastern strain)..... | Above 4.1 | Negative | Finkelstein, Marx, Bridges and Beard |
| Vesicular stomatitis..... | 6.6-8.4 | Negative | Olitsky and Cox (personal communication) |

3. *The electrical charge of antibodies.* The electrical charge of purified pneumococcal antibodies has recently been investigated by Felton, Felton and Kauffman, Chow and Goebel and Green, McKhann, Kupnik and Fakey. The isoelectric points ranged from pH 7.0 to 7.4 conforming with the fact that the capillaries of the C-N-S are permeable to antibodies.

4. *The electrical charge of drugs.* It is remarkable that all drugs to which the capillaries of the C-N-S have been shown to be impermeable, namely, neoarsphenamine, Bayer 205, the anions of the ferrocyanides, iodides, bromides and thiocyanates are negatively charged. On the contrary, the alkaloids are elec-

tropo-positive substances. They act on the C-N-S and, at least, some of them have been definitely shown to pass the capillaries of the C-N-S.

5. *The electrical charge of spirochaetes and trypanosomes.* There is no doubt that *Treponema pallidum*, the spirochaetes of recurrent fever (Busehke and Kroo) and *Trypanosoma gambiense* and *rhodense* may pass the capillaries of the C-N-S. Omitting older investigations carried out with a questionable technique we wish to point to the careful cataphoresis experiments of Broom, Brown and Hoare with various strains of trypanosomes. It was found that some of them carried positive and others negative charges. With the same strain a change in the charge was observed after a relapse and after treatment with arsenicals. Thus far, no attempt has been made to correlate the electrical charge of these organisms with their ability to pass the capillaries of the C-N-S. Such investigations would be of great interest in connection with the much discussed problem of the existence of neurotropic strains of *Treponema pallidum* (Lévaditi and Marie).

Brown and Broom made another interesting observation. They found that electropositive trypanosomes conglomerated with the negatively charged erythrocytes while electronegative trypanosomes failed to do so. A similar conglomeration of trypanosomes with the capillary endothelium may be a prerequisite for their ability actively to invade the C-N-S.

These investigations on the electrical charge of aniline dyes, toxins, viruses, antibodies and drugs seem to indicate that the ability of substances to pass the capillaries of the C-N-S may be explainable on an electrochemical basis. In section XI some theoretical aspects of this concept will be discussed.

X. *The permeability of other capillaries to polar substances.* The experiments of Goldmann, Rous, Gilding and Smith, Singer (88) and Schulemann have shown definitely that the capillaries in other organs are permeable to acid aniline dyes. In the choroid plexus (Wittgenstein and Krebs, 161a) ciliary plexus (Fischler) and peritoneum (Engel and Kerekes) the capillaries are even impermeable to basic dyes. Moreover, there can be no doubt that in some tissues the capillaries are permeable to the negatively charged tetanus, diphtheria, botulinus and staphylococcus toxins. According to Wallae et al. the capillaries in all organs excepting the brain are perfectly permeable to the anions of bromides, iodides and thiocyanates.

Nevertheless, Wittgenstein and Krebs (161b) have shown that the capillary system as a whole, although permeable to acid dyes, is much more readily passed by basic dyes. They found that the latter disappeared far more rapidly from the circulation than acid dyes. Some colloidal acid dyes, for instance water blue, could be identified in the blood plasma for many weeks. The same holds true for the electronegative drug Bayer 205.

It appears, therefore, that the complete impermeability to electronegative substances is a peculiarity of the capillaries of the C-N-S. The difference between these capillaries and those in other organs, however, is more of a quantitative than a qualitative nature. This difference can be explained on a variety of assumptions. We wish to point to the fact that according to Overton (122)

the permeability to basic and the impermeability to acid dyes is characteristic of cell membranes in general. It would be tempting, therefore, to assume that in the capillaries of the C-N-S the exchange takes place predominantly if not exclusively through the capillary endothelium whereas in other organs the intercellular cement substance (W. Zweifach) may be of greater importance. Whether this hypothesis can be substantiated by histological methods remains for further investigations.

XI. *Theoretical considerations.* It must be admitted that our conceptions are at variance with Overton's theory on the permeability of cell membranes. According to this author cell membranes are impermeable to ions. The vital staining properties of basic dyes are not due to their positive electrical charge but to the lipid solubility of the undissociated dye bases. At the Symposium on permeability at Cold Spring Harbor (1940), however, the majority of participants arrived at the conclusion that cell membranes are permeable to ions. Moreover, the experiments of Mandelstamm and Friedemann (58) have definitely shown that the ability of basic dyes to pass the cerebral capillaries is independent of their lipid solubility.⁴

Our point of view is further strengthened by the discovery of Michaelis that dried collodium membranes are permeable to cations but impermeable to anions. Although not of a lipid nature, therefore, they resemble the capillaries of the C-N-S.

An irreciprocal selective permeability to ions was described by Wertheimer in the skin of the frog. This membrane is passed by basic dyes only from the inner to the outer surface and by acid dyes in the opposite direction. Wertheimer explains these results by assuming an asymmetrical structure of the skin with a resulting electrical potential gradient through the membrane.

A selective irreciprocal permeability to ions would follow also from the well known theory of the Donnan equilibrium. Actually King has tried to explain Goldmann's experiments with trypan blue in this way. Since it has been shown in section IV that the cerebral capillaries are impermeable to acid dyes, the site of the Donnan equilibrium should be the capillary membrane itself. In this case, however, the cerebral capillaries should be permeable to acid and impermeable to basic dyes in the direction from blood to tissue. For at the pH of the blood the proteins are negatively charged and their concentration in the blood plasma is more than 20 times higher than in the tissue fluid.

In this connection mention must be made also of the extensive work of Keller and his associates which is entirely based on an electro-chemical conception. In his opinion the distribution of substances in animals and plants is controlled by electrostatic forces in the tissues. To adapt his theory to experimental facts Keller assumes that the electrical charge of aniline dyes in the tissues and the body fluids is entirely different from that in water. Keller and Singer have published a table in which aniline dyes are classified on the basis of their "bio-

⁴ In the opinion of the author, the experiments of Nirenstein cannot be considered as evidence for the theory of lipid solubility. On the contrary, they point very strongly to the importance of electro-chemical factors.

logical charges". A glance at their table shows that this classification is in no way related to our experimental results on the permeability of the cerebral capillaries. Although I agree with Keller in the electrochemical aspect of the problem of distribution, I cannot accept some details of his theory.

This discussion shows that the selective permeability of the capillaries of the C-N-S to electropositive or electroneutral substances can be explained by a variety of theories. The important question of the reciprocity of the permeability of the capillaries to polar substances will be discussed further in section XIII.

XII. *Some toxicological problems.* In the preceding sections we have seen that the capillary system as a whole is more permeable to electropositive than electronegative substances. The same holds true for the majority of cell membranes. A relationship between the electrical properties of substances and their toxicity, therefore, presents itself as a possibility. We cannot review in this context the entire field of pharmacology. Our surmise, however, finds support in the high toxicity of the electropositive basic aniline dyes, alkaloids, biogenic amines and ammonium bases and the relative atoxicity of the electronegative acid aniline dyes, neoarsphenamine, Bayer 205 and potassium ferrocyanide.

Regarded from this point of view, toxins are of particular interest. At first sight no relationship seems to exist between their electrical and toxic properties. Despite their negative charges tetanus, diphtheria and botulinus toxins are just as toxic as cobra venom and lamb dysentery toxin. The electrical character, however, is reflected in the rapidity of action. The three electronegative toxins act only after an incubation period of one or several days. Electropositive cobra venom and electroneutral lamb dysentery toxin, however, kill susceptible animals within a few minutes. In conformity with our theoretical expectation it has been found that the two groups of toxins differ markedly in the velocity with which they pass the capillary walls. After intravenous injection tetanus, diphtheria and botulinus toxins can be identified in the blood for many hours. Cobra venom and lamb dysentery toxin, however, leave the vascular system almost immediately. This follows from the fact that their lethal dose is considerably higher by the intra-arterial than the intravenous route. This has been shown by Straub for the toxin of an anaerobic bacillus probably closely related to lamb dysentery toxin and by us for cobra venom and lamb dysentery toxin (unpublished experiments). Apparently a large proportion of these toxins leaves the vascular system during a single circulation through the capillary bed. In addition, however, the rapid action of these toxins must be due to the velocity with which they pass cell membranes or are absorbed at their surface. Otherwise it would not be explainable that these toxins act rapidly also when injected directly into the brain (Friedemann and Elkeles 65).

Our explanation of the length of the incubation period of toxins, apparently, does not apply to the experiments with staphylococcus toxin which combines a rapid action with a negative charge at the pH of the blood. Cataporesis

experiments of Friedemann (58) which cannot be described in detail, seem to indicate that it may be more correct to correlate the incubation periods of toxins with the ξ -potential than with the sign of the electrical charge. This would have the indubitable advantage of relating gradations of permeability to a graded electrochemical factor. Actually the ξ -potential of staphylococcus toxin was found to be low as compared with that of diphtheria toxin. It is intermediate between the ξ -potentials of lamb dysentery toxin and cobra venom on the one side and electronegative toxins on the other. If this conception can be substantiated by further experiments it would lead to the important conclusion that permeation of polar substances through the capillary walls is a cataphoretic phenomenon and, consequently, irreciprocal.

XIII. *The pathology of the blood-brain barrier.* Although we have seen that under normal conditions the C-S-F has no part in the exchange of substances between blood and C-N-S this may be different if the choroid plexus or the meningeal vessels are damaged. On the basis of the theories of v. Monakow and Stern even the mere presence of constituents of the blood and especially of proteins in the C-S-F might be detrimental to the C-N-S. Along these lines Weil and Kafka have advanced a theory of the pathogenesis of general paresis.

They found, by the hemolysin method, that in this disease the C-S-F has a high protein content and considered this factor responsible for the degenerative changes in the C-N-S. Hauptmann elaborated a similar theory for the pathogenesis of tabes dorsalis. The now established fact, however, that the normal cerebral capillaries are permeable to proteins deprives these theories of their theoretical foundation.

An increased permeability of the capillaries of the C-N-S under pathological conditions has been reported only by Faber. He found that in monkeys infected with poliomyelitis virus the C-N-S was stained by intravenously injected trypan blue.

In young mice the capillaries of the C-N-S are apparently not entirely impermeable to electronegative substances. This was demonstrated with trypan blue by Behnsen and with the neurotropic strain of yellow fever virus by Theiler. The "Kernicterus" of the newborn may point in the same direction.

An increased permeability of the capillaries of the C-N-S was artificially produced by Froehlich and Zak with theophylline, by Friedemann and Elkeles (63) with adrenaline and by Duran-Reynals with testicular extracts (spreading factor). In the experiments with adrenaline and testicular extract the increased permeability concerns only those substances to which the capillaries of the C-N-S are normally permeable. Froehlich and Zak found that in the frog theophylline rendered the cerebral capillaries more permeable also to some acid dyes. The investigations of Barbour and Abel and Macht as well as our own experiments, however, have shown that in the frog the cerebral capillaries are normally permeable to a number of acid dyes. The observation of Froehlich and Zak that after the application of theophylline intravenously injected potassium ferrocyanide was found in the brain requires further analysis. We leave the question open whether theophylline makes the cerebral capillaries permeable to such substances to which they are otherwise impervious.

A traumatic breakdown of the blood-brain barrier has been performed by McCurdy and Evans, Macklin and Macklin, Mendel, Morgenstern and Birjukow and Broman. The traumatized area was found stained by intravenously injected trypan blue. Mendel and more recently King explain these results on a chemical basis. They assume that traumatized brain tissue has an affinity for trypan blue while normal tissue has not. Experiments with neurotropic viruses, however, make this explanation appear unlikely. Flexner and Amoss, Lennette and Hudson, Zwick, Scifried and Witte, Sawyer and Lloyd, Galloway and Nicolau, Burnet and Lush, and Webster and Clow have shown that the intracerebral injection of sterile saline, serum or starch renders animals susceptible to the intravenous, intraperitoneal or intracutaneous injections of a variety of neurotropic viruses which are otherwise innocuous. Since these viruses are extremely pathogenic for the untraumatized C-N-S these results can be explained only by the effect of the trauma on the permeability of the cerebral capillaries.

SUMMARY

Experiments with aniline dyes, toxins, viruses and drugs have definitely established the existence of a barrier between blood and C-N-S. This barrier is localized in the capillaries of the C-N-S which are endowed with a selective permeability. The choroid plexus, meningeal vessels and C-S-F are not concerned with the exchange of substances between blood and C-N-S.

The permeability of the capillaries of the C-N-S has now been determined for a considerable number of aniline dyes, toxins, viruses, antibodies and drugs. As far as the experimental evidence goes it seems to indicate that the ability of substances to pass the capillaries of the C-N-S is determined by their electrochemical properties. The cerebral capillaries are permeable to substances carrying a positive or no charge at the pH of the blood while they are impermeable to those carrying a negative charge. The permeability of the capillary system as a whole may be more correctly correlated to the ξ -potential than to the sign of the electrical charge.

The capillaries in the majority of organs are permeable to electronegative substances but they are more readily passed by the electropositive ones. The choroid plexus, ciliary plexus and the capillaries of the peritoneum are permeable to acid (electronegative) and impermeable to basic (electropositive) dyes.

The higher permeability of the capillary system as a whole to electropositive substances explains the greater toxicity of basic drugs and the rapid action of cobra venom and lamb dysentery toxin in contradistinction to the slow action of tetanus, diphtheria and botulinus toxins. These investigations have shown that toxins, viruses and antibodies can be used for the study of physiological problems, while, on the other hand, the physiological approach to bacteriological and immunological problems has led to some important results. The distribution of toxins between blood and C-N-S and differences in the lengths of incubation periods have been explained from the point of view of permeability and on an electrochemical basis.

In concluding this paper it must be emphasized that the term "blood-brain

barrier" is no longer entirely adequate. It does not take account of the fact that the problem is only part of the more general problem of capillary permeability and it places too much emphasis on its negative aspect. Nevertheless the term has been retained in the title, partly for historical reasons, and partly because the impermeability of the cerebral capillaries to a variety of pathogenic agents is important for many questions of pathogenesis which concern the C-N-S alone.

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CYTOLOGICAL ASPECTS OF SYNAPTIC FUNCTION

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It is not difficult to anticipate a phase of investigation of the synapse in which recent refinements in histological and histochemical methods, combined with the already highly developed neurophysiological techniques, may produce new data of great importance in our understanding of the mechanisms of synaptic function. Attempts at closer synthesis of physiological and histological data, however, although actively in progress,² are still rendered difficult by the obstacles encountered in adequately preserving and staining synaptic terminals in the nervous system. These difficulties not only hinder the determination of needed histochemical and histophysiological data, but have made even the amassing of purely descriptive morphological data a relatively slow process. Nevertheless, a not inconsiderable body of relevant anatomical facts exists which has not thus far been summarized and made conveniently available for those who are interested but who have perhaps no direct experience with histological material. One of the primary aims of this account will therefore be to emphasize certain aspects of the anatomy of the synapse which seem to the reviewer to require more attention in general considerations of synaptic physiology, and which may suggest specific problems for future investigation.

The physiological evidence bearing on particular aspects of synaptic function has been reviewed frequently in recent years³ and will only partly fall within the scope of this discussion. In fact, only a small part of the evidence from anatomy can be summarized in this review, for the essence of the organization of the nervous system, and especially of synapses, lies in the complexity of arrangement of parts and the diversity of structure of the parts themselves.

For additional literature on the morphology of synaptic endings, the reader may be referred to the final summary of the eminent Spanish histologist, Ramón y Cajal (1934).

GENERAL HISTOLOGICAL DETAILS. Although half a century has elapsed since the pioneer histological studies of synaptic axon endings⁴ the methods used in such studies have changed during that period only in the direction of refinement.⁵ The controversy on the neuron doctrine and on the problems of protoplasmic and neurofibrillar continuity at the synapse, which often occupied earlier neuro-

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² Lorente de Nó, 1935, 1935a, 1938, 1938a, 1939; Young, 1939; O'Leary, 1940; Renshaw, Forbes, and Morison, 1940; Lloyd, 1941; and others.

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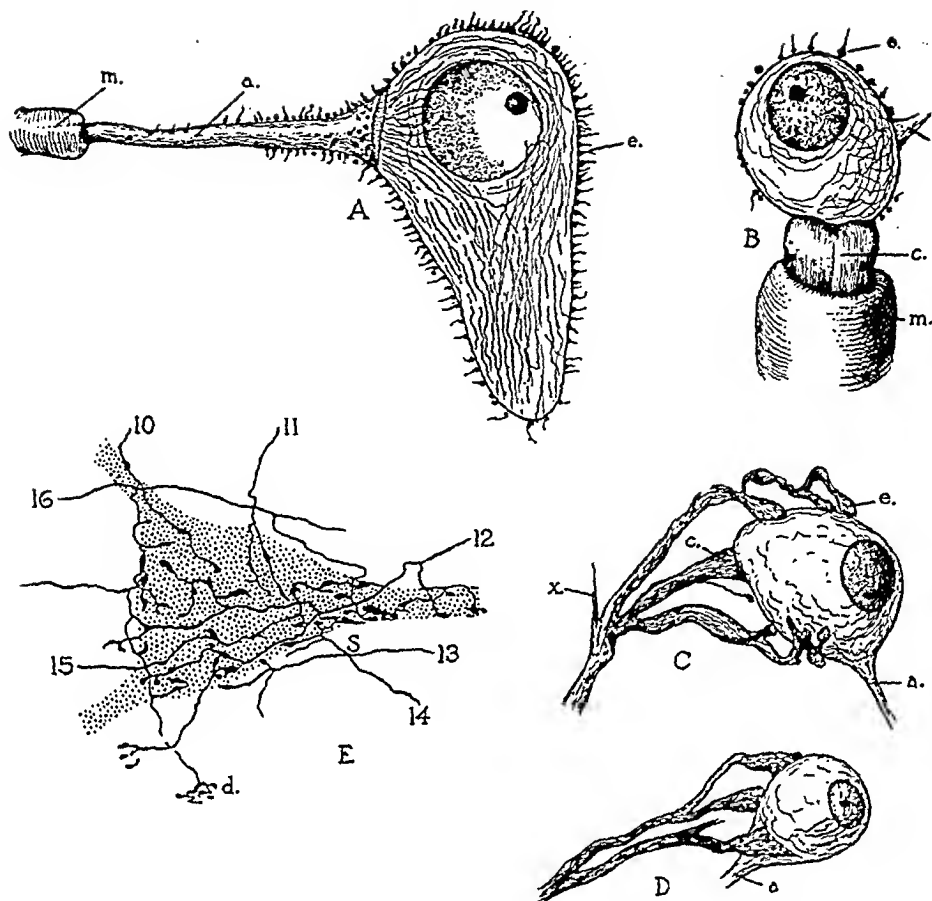
histologists to the exclusion of any other interest in synaptic structure is still waged in some quarters.⁶ However, the evidence for the existence of a membranous synaptic barrier, not only in vertebrates but in some invertebrates as well, has become so voluminous that much investigation has turned toward other important aspects of synaptic structure. At the present time, and especially for purposes of functional analysis, it is desirable that more be known concerning problems such as the ultrastructure of the membranes concerned, the quantitative relations existing between surfaces of axon terminals and the surfaces of cells of termination, the intimate relationships of myelin and of glia to synaptic surfaces, the types, approximate numbers, and specific sites of origin of endings on cells of each type, the electrical properties of cellular elements and intercellular materials, and the localized concentrations and site of action of both drugs and physiological substances which may produce or modify neuronal excitation, under varying conditions. Some of these problems await the further development of microphysiological and histochemical methods for adequate analysis. Investigation of the purely morphological problems is also attended with great difficulties, because many axonal terminals are so minute and their post-mortem change is often very rapid.⁷ The uncertain staining qualities of axon endings, because of these and other factors, constitute the most serious obstacle in the determination of normal anatomical relationships as well as of experimentally induced changes. No method has yet been devised to study nerve cells and their synapses in the central nervous system in the living state, although an important study of sympathetic ganglion cells and their synapses has been made in vital preparations of the frog by Fedorow (1935), and Speidel (1936) has studied living cutaneous axon end bulbs. The findings obtained from these studies of living material agree well with those described in suitably prepared histological sections (Bodian, 1940). Unfortunately, however, it has been impossible by present histological methods to demonstrate synapses with consistency in some centers where they are known to be present, and, in other centers, such as the mammalian thalamus, in which abundant terminal axon arborizations have long been known (Ramón y Cajal, 1911), details of the intimate relationship of the axon terminals to the cells of termination have only recently been described (Glees and Clark, 1941; Glees, 1941). These facts compel caution in generalizing from those synaptic bulbs which are more easily stained, although the great variety of the latter, coupled with their possession of certain characteristics in common, permits a tentative summary of their elementary characteristics as follows:

Synaptic endings as axon enlargements. Most axons and their collaterals terminate by means of protoplasmic enlargements (Ramón y Cajal, 1893) which serve to increase the terminal contact surface. The most easily and universally demonstrable type of synaptic axon ending is the endfoot or bouton (figs. 1 and 2). They are similar in form in all vertebrates,⁸ as well as in invertebrates

⁶ Nonidez, 1937; Nageotte, 1938; Boeke, 1940, 1941.

⁷ Ramón y Cajal, 1903; Marinesco, 1904a; Bartelmez, 1915; Bartelmez and Hoerr, 1933; Fedorow, 1935; Speidel, 1936; Bodian, 1937, 1940.

⁸ Ramón y Cajal, 1934; Phalen and Davenport, 1937; Bodian, 1937.



Figs. 1 and 2. A series of nerve cells showing various types of synaptic systems, and the distribution of axon endings on the surface of the terminal neuron.

Fig. 1. A. Large motor type cell from the reticular formation of the goldfish showing relatively uniform distribution of homogeneous boutons, *c*, on soma and proximal part of axon, *a*. From an 8μ section, Mallory-azan stain. $\times 960$. This type of synaptic system is the most common in the vertebrate nervous system and is characteristic of motoneurons and interneurons. One manner in which the synaptic knobs may be related to parent fibers is shown in E, a large interneuron from the spinal cord of a 15 to 16 day old cat; Golgi method. (Redrawn from Lorente de N6, 1938.)

B. Cell of reticular formation of goldfish showing in addition to small boutons, *e*, a single large club ending, *c*, of a myelinated axon, *m*. Such club endings on small cells are in contact with a relatively large part of the cell surface. From an 8μ section, Mallory-azan stain. $\times 960$.

C and D. Two cells of the oculomotor nucleus of the goldfish, showing distinctive basket-like system of endings (knob and club-like units) derived from a single large branching axon. The general pattern of such endings on all cells of this nucleus is similar but the details of branching and distribution of endings on the cell surface differ in each case. From 15μ section. Bodian stain. $\times 960$. Similar synaptic systems are found in many centers in the nervous systems of vertebrates (see page 157).

a, axon of cell of termination. *c*, club ending. *e*, cndbulbs or boutons terminaux. *m*, myelin sheath. *x*, collateral fiber.

(Sereni and Young, 1932; Young, 1939), and their size ranges vary from about $\frac{1}{2} \mu$ to 5μ , and as large as 7μ .⁹ Nevertheless, such boutons, although repre-

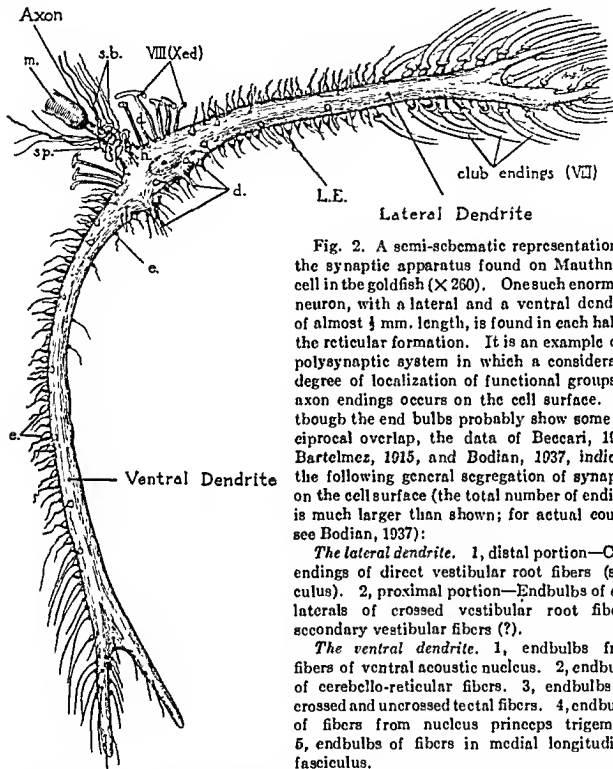


Fig. 2. A semi-schematic representation of the synaptic apparatus found on Mauthner's cell in the goldfish ($\times 260$). One such enormous neuron, with a lateral and a ventral dendrite of almost $\frac{1}{2}$ mm. length, is found in each half of the reticular formation. It is an example of a polysynaptic system in which a considerable degree of localization of functional groups of axon endings occurs on the cell surface. Although the end bulbs probably show some reciprocal overlap, the data of Beccari, 1907; Bartelmez, 1915, and Bodian, 1937, indicate the following general segregation of synapses on the cell surface (the total number of endings is much larger than shown; for actual counts see Bodian, 1937):

The lateral dendrite. 1, distal portion—Club endings of direct vestibular root fibers (sacculus). 2, proximal portion—Endbulbs of collaterals of crossed vestibular root fibers; secondary vestibular fibers (?).

The ventral dendrite. 1, endbulbs from fibers of ventral acoustic nucleus. 2, endbulbs of cerebello-reticular fibers. 3, endbulbs of crossed and uncrossed tectal fibers. 4, endbulbs of fibers from nucleus princeps trigemini. 5, endbulbs of fibers in medial longitudinal fasciculus.

The perikaryon. 1, small endbulbs on axon hillock derived from spiral fibers (s.b.) of the medial longitudinal fasciculus (from mesencephalic nucleus of the medial longitudinal fasciculus (?)). 2, knob endings of collaterals of crossed vestibular root fibers (VIII (xed)) near axon hillock (some uncrossed fibers (?)). 3, endbulbs of fibers in medial longitudinal fasciculus. 4, endbulbs of secondary acoustico-lateral fibers (?).

d—small dendrites. e—small endbulbs. h—axon hillock. L.E.—large endbulbs. m.—myelin sheath of axon of Mauthner cell. s.b.—bundle giving origin to spiral fibers. sp.—spiral fibers in region of "axon cap". VIII (xed)—crossed vestibular fibers giving rise to collaterals which terminate as small club endings. VIII—vestibular root fibers.

⁹ Sereni and Young, 1932; Hoff, 1932; Phalen and Davenport, 1937; Bodian, 1937; Barr, 1939; Gibson, 1940; Minckler, 1940.

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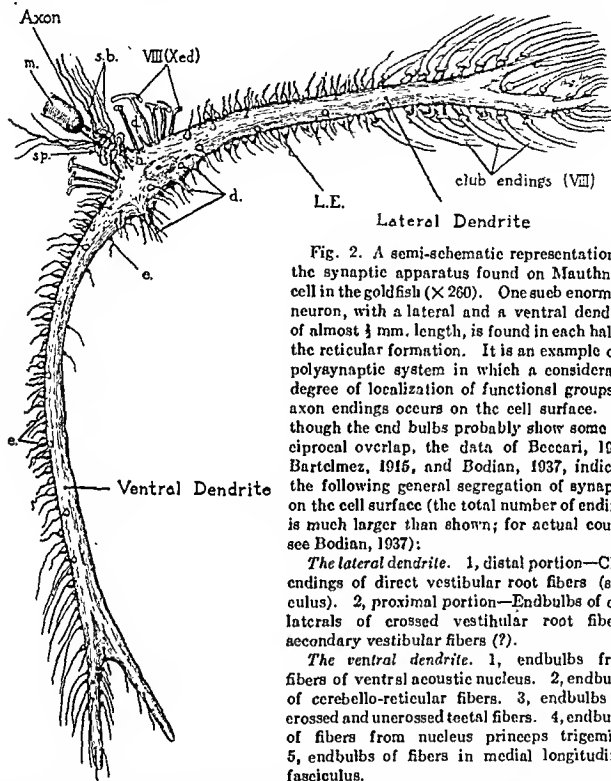


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The perikaryon. 1, small endbulbs on axon hillock derived from spiral fibers (s.b.) of the medial longitudinal fasciculus (from mesencephalic nucleus of the medial longitudinal fasciculus (?)). 2, knob endings of collaterals of crossed vestibular root fibers (VIII (xed)) near axon hillock (some uncrossed fibers (?)). 3, endbulbs of fibers in medial longitudinal fasciculus. 4, endbulbs of secondary acoustico-lateral fibers (?).

d—small dendrites. e—small endbulbs. h—axon hillock. L.E.—large endbulbs. m.—myelin sheath of axon of Mauthner cell. s.b.—bundle giving origin to spiral fibers. sp.—spiral fibers in region of "axon cap". VIII (xed)—crossed vestibular fibers giving rise to collaterals which terminate as small club endings. VIII—vestibular root fibers.

⁹ Sereni and Young, 1932; Hoff, 1932; Phalen and Davenport, 1937; Bodian, 1937; Barr, 1939; Gibson, 1940; Minekler, 1940.

senting the workaday prototypes of the synaptic axon endings, typify only one of several forms of such endings, and may occur alone or with other types on the same cell (fig. 1, B; fig. 2). The club endings of Bartelmez¹⁰ (fig. 2) and certain endings in invertebrates¹¹ do not possess terminal swellings, but since these are relatively large fibers, it may be supposed that little can be accomplished by further enlargement of the axon at its contact surface. Non-bulbous "free endings" of fine fibers in the central and peripheral nervous systems would also seem to fall into this category. However, the vagaries of the histological methods used in demonstrating such fibers suggest the possibility that their actual terminations have not really been demonstrated.

The synaptic membrane or "synaptolemma." When the tissue has been well preserved, preferably by perfusion of the living animal, and stained by adequate cytological methods, synaptic enlargements are seen to be separated from the cell of termination by a sharply stained membrane.¹² At these endings only one membrane can be resolved, presumably because of the intimacy of contact of the separate neuronal limiting membranes. This appositional membrane, which we may speak of as the "synaptolemma" for convenience, separates cytoplasmic masses which have different staining properties as a rule, and is characteristic of synapses formed by boutons, club-shaped endings, and calycoform endings in vertebrates and in invertebrates. Whether a similar synaptolemma is present in the region of approximation of more complex axodendritic arborizations, as in the olfactory and cerebellar glomeruli of vertebrates, is not so clear, since such structures have been analysed only by silver impregnation methods. Unfortunately such methods, even in experienced hands, are difficult to control and give incomplete pictures which are not easily interpreted (Bartelmez and Hoerr, 1933; Bodian, 1937).

Young (1936, 1939) has described an interesting case of protoplasmic fusion of the axons of the two first order giant cells of the squid. These cells, however, are associated with other nerve cells on both the afferent and efferent sides by typical contact synapses. This case of fusion of axoplasms of two cells, and other cases cited by him, he has interpreted as secondary fusion of axons which "must always work together during the life of the animal." The fusion joins parallel synaptic systems, at points which would not ordinarily have been synaptically related, namely, a point of decussation of homologous giant fibers. Various degrees of intimacy of contact and of fusion of giant fibers at the point of decussation have been described by Hamaker (1898) in Nereis and by Johnson (1923) in crustaceans, and the latter author has suggested that this close contact, and in some cases fusion, of decussating fibers may permit passage of nervous impulses from one to the other for coordination of movements of the two sides of the body. In the cephalopods, Young (1939) states that this synapse-like relationship is apparently secondarily abolished by complete fusion, and other cases of fusion of parallel or homologous axons in invertebrates to form giant fibers have been described.¹³ A somewhat analogous example of close association of parallel axons of homologous function within a single sheath has been described by Holmgren and van der

¹⁰ Bartelmez, 1915; Bartelmez and Hoerr, 1933; Bodian, 1937, 1940.

¹¹ Johnson, 1923; Stough, 1926; Young, 1939.

¹² See ¹⁰ and Young, 1939.

¹³ Hamaker, 1898; Lewis, 1898; Johnson, 1923; Young, 1939; Cardot and Arvanataki, 1941.

Horst (1925) in the lungfish *Ceratodus*, but these writers doubt that the occasional points of fusion of these axons which they observed was not artefactual. The conduction properties of this interesting fascicle remain to be investigated.

Instances of protoplasmic fusion of dendrites have also been described (Dogiel, 1893; Glees, 1938), but it is doubtful that this is other than exceptional, and here the fusion does not abolish synaptically related surfaces. Such cases, although apparently breaking down the universality of application of some of the tenets of the neuron doctrine, do not in any way invalidate the evidence for the predominance of synaptic systems in all vertebrates, as well as in many of the invertebrates.¹⁴

Mitochondria at the synapse. On the proximal or afferent side of the synaptotlemma an accumulation of mitochondria-like granules has been described (Bartelmez, 1915; Bartelmez and Hoerr, 1933). This was confirmed by Bodian (1937, 1940) for the club endings and also for large and small endings of the bouton type in the goldfish, as well as in the cephalopods (fuchsinophilic granules) by Young (1939). These granules are probably the neurosomes described by Held in 1897, although he failed to preserve the entire structure of the end-foot. It is noteworthy that in the "boutons" of the cardiac ganglia of the living frog, Fedorow (1935) has found that granules which stain with Janus green and with neutral red increase in number following stimulation of the preganglionic fibers. Concentrations of mitochondria in the synaptic cerebellar glomeruli have also been described by Held (1897) and by Ortiz-Picon and Pérez-Lista (1929). The function of these granules is unknown, but their aggregation near the synaptic membrane is a challenge to histochemistry. This aggregation might be interpreted as being correlated with a local secretion of acetyl choline or of choline esterase, especially since the latter appears to be associated with the surfaces at ganglionic synapses as well as at neuromuscular junctions (mysynapses), according to Couteaux and Nachmansohn (1940). On the other hand the aggregation of mitochondria might simply represent an anatomical signature of more general metabolic processes concerned in locally modifying the electrical properties of the membrane.

Neurofibrils at synaptic junctions. In all cases where a synaptotlemma can be demonstrated, the neurofibrils of the two elements of the synapse are distinct, and do not cross the synaptic membrane.¹⁵

"Regulation" and regeneration of synaptic endings. Synaptic end-bulbs bear considerable resemblance to peripheral axonal endings, both sensory and motor, and all may be considered as stemming from the embryonic growth cone (Ramón y Cajal, 1890; Harrison, 1910). Such synaptic bulbs have also been described in cultures of embryonic chick brain.¹⁶ The ability of peripheral axon endings in tadpoles to adjust to injury by forming new endings with different arborization patterns has led Speidel (1941) to suggest the possibility of a similar regulatory capacity of the central boutons. This notion is placed in serious questions by the fact that mature central axons possess notoriously slight capacities

¹⁴ Schafer, 1878; Bozler, 1927; Hanström, 1923; Sereni and Young, 1932; Young, 1939; Woolard and Harpman, 1939; Abraham, 1940; Bullock, 1940.

¹⁵ See ¹⁰, Ramón y Cajal, 1934; and others.

¹⁶ Bauer, 1932; Mibálik, 1932; Grigorjeff, 1932.

for regeneration, and by the absence of clear evidence that mature central boutons ever retract, even during the period either of axon reaction of the terminal cells,¹⁷ or of actual degeneration of the terminal cells (discussed by Ramón y Cajal, 1934). The tendency for boutons to adhere firmly to the terminal neuron, so as to resist separation by shrinkage in fixed preparations, has been noted frequently, and a high degree of stickiness has also been observed in teased preparations by Carpenter (1911). Regeneration of terminal boutons in sympathetic ganglia after degeneration following nerve section has however been described,¹⁸ as well as coincident restoration of function in the case of the superior cervical ganglion.¹⁹ In various reports on anatomical and functional regeneration of nerve fibers in the central nervous system, the fate of the terminal synaptic apparatus has not been considered (see Sugar and Gerard, 1940, for literature).

Degeneration of synaptic endings. Early suggestions as to the susceptibility of synaptic boutons to pathological states occurring, for example, in experimentally induced ischemia of the spinal cord (Marinesco, 1904b), and in rabies (Ramón y Cajal, 1904) have thus far led to no serious investigations of alterations of these structures in subtler pathological conditions. The difficulties of such investigations are obvious. The studies of experimental changes occurring in axon end-bulbs after axon section, although not as yet adequately exploited, have yielded more abundant fruit. Such studies, first carried out in sympathetic plexuses and ganglia,²⁰ have demonstrated conclusively the reality of the synaptic knobs as interneuronal junctions, and have opened up the possibility of histological controls for degeneration studies of synaptic physiology (Gibson, 1940). Although similar degenerative phenomena of synaptic boutons have now been described in the central nervous system by many investigators,²¹ it remains to be seen whether the hoped-for precise determination of axonal connections by this method will be attained. Such a demonstration, to be rigorous, must surmount the difficulties presented by the great numbers of boutons on many nerve cells, often from many sources, the vagaries of the silver impregnation methods used in these investigations, resulting usually in incomplete staining of boutons in most instances, and the problem of distinguishing with absolute certainty the altered boutons from normal ones. Because of these difficulties, some of the claims made for this method have been criticized by Barnard (1940), who with Schimert (1938, 1939) has made clear the need for careful operative and histological control. Moreover, the validity of Hoff's findings (1932a, b) that pyramidal tract endings occur on motoneurons in the cat, and dorsal root endings do not, has recently been rendered questionable,

¹⁷ Lawrentjew, 1934; Barr, 1940; Schadewald, 1940. Contra, Barnard, 1940.

¹⁸ Lawrentjew, 1925, 1934; De Castro, 1930; Gibson, 1940.

¹⁹ De Castro, 1930, 1936; Gibson, 1940.

²⁰ Nikolajew, 1893; Lawrentjew, 1924, 1925, 1934; De Castro, 1930, 1934, 1936; Kolossow, 1932; Kolossow and Sabussow, 1932; Kolossow, Sabussow and Iwanow, 1932; Baron, 1934; Fedorow, 1935; Fedorow and Matwejewa, 1935; and Gibson, 1940.

²¹ Hoff, 1932a, b, 1935; Hoff and Hoff, 1934; Foerster, Gagel and Sheehan, 1933; Gibson, 1937; Schimert, 1938, 1939; Snider, 1936; Rosiello, 1937; Glees, 1941; Glees and Clark, 1941.

for example, by the contrary physiological findings of Lloyd (1941) and Renshaw (1940), respectively.

Transneuronal effects of axon section, consisting of cellular atrophy and extending beyond the synaptic junction, are implicit in the degeneration method of Gudden, and have in recent years been described as occurring after several months in adult individuals, especially in the lateral geniculate body following optic nerve section in primates.²² Similar changes have been described in the spinal cord after root section by Foerster and Gagel (1934), who however were probably dealing with vascular injury. The transneuronal atrophy does not occur in all centers but probably only in those in which the severed afferent fibers form the bulk of the incoming nerve fiber supply. Ignorance of the mechanism of this atrophy is indicated by the suggestion that it may be considered as a "disuse atrophy". However, the increased irritability to chemical agents of denervated structures (Cannon, 1939) suggests a possible analogy to the "fatigue" atrophy of fibrillation in denervated skeletal muscle (Tower, 1939). Perhaps several factors determine the occurrence and degree of atrophy in deafferented neurons, just as in denervated muscle (Tower et al., 1941 a, b).

The synaptic environment. Since the region of the synapse is functionally of such great importance, it is necessary to inquire into the relationships of the various histological constituents of this region. Of the structures which may, by virtue of proximity and specific metabolism, affect the functioning of the synapse, the blood vessels, tissue fluid, neuroglia cells, and nerve fibers must be considered, but little can be said regarding the rôle of non-neuronal structures except that the close investment of synaptic structures by glial processes may be of some functional significance. An analogous investment of axon endings in muscle is suggested by the work of Noël and Pommé (1932), who consider the sole plate to consist of cellular elements enveloping the axon endings and continuous with and derived from the sheath cells of the terminating axon. Since Couteaux and Nachmansohn (1940) have found that in the region of the sole plates of mammalian gastrocnemius muscle an initial decrease of choline esterase after denervation is followed by the persistence of this substance in high concentration (60 per cent), it might be supposed that the supporting cells at effector endings play an important rôle in the function of myosynapses. This immediately suggests a similar rôle of glia cells in the functioning of neurosynapses, but although Couteaux and Nachmansohn have found a similar persistence of choline esterase in the superior cervical ganglion after preganglionic nerve section, the quantity involved was considerably less than in muscle and might have been localized within the nerve endings of undegenerated recurrent collateral fibers. Regardless of the interpretation of these data, however, it is evident that the conception of neuroglia cells as passive supporting structures is inadequate, since these cells may be of importance not only in the chemical economy of nervous tissue but also in the conditioning of its electrical properties. Indeed it is not inconceivable that the suggested varying importance of chemical as contrasted with bioelectrical mechanisms in synaptic transmission in differ-

²² Minkowski, 1920; Balado and Franke, 1930; Clark and Penman, 1934.

ent nerve centers²³ may be dependent on functional differentiation of neuroglia cells or analogous cells in the peripheral nervous system.

Passing unmyelinated nerve fibers often thickly encompass a nerve cell or its dendrites, but such fibers have not been seen in actual contact with the surfaces of nerve cell bodies or of dendrites, except by means of synaptic swellings (L. de No, 1938). On some nerve cells, where endings are especially numerous, the passing fibers come no closer to the cell membrane than the diameter of the terminal bulbs, so that a fiber-free zone, not to be confused with shrinkage artifacts, can often be seen to surround the perikaryon or dendrite (Bodian, 1940). However, in some instances the fibers of the surrounding neuropil may press very closely upon a portion of a cell body or dendrite, and in some such cases one may see no terminal bulbs or any other type of synaptic structures upon this part of the cell surface (compare medial and lateral surfaces of ventral dendrite of Mauthner's cell, fig. 2). In such cases, as also in the case of the cerebellar climbing fibers and "spiral" terminal fibers such as seen in the sympathetic ganglia²⁴ and around the Mauthner cell axon hillock (Bodian, 1940), it is conceivable that unmyelinated fibers may have a functional influence upon adjoining nerve cells or their processes. Whether in the vertebrate neuropil such influences of adjoining unmyelinated fibers are of the nature of subthreshold excitability changes resulting in synchronization as described by Katz and Schmitt (1940) in crab nerves, or by Blair and Erlanger in the frog (1940), or are even capable of transmitting a nervous impulse (Jasper and Monnier, 1938; Renshaw and Therman, 1941) is conjectural. In the event of possible synapse by axonal apposition, such as the case of climbing fibers in the cerebellum, of granule cell collaterals of the cerebellum, and perhaps also of some of the fibers terminating on sympathetic ganglion cells, the existence of contact points or actual synaptic thickenings must seriously be considered (Lawrentjew, 1924; Ramón y Cajal, 1934).

Whenever pre-synaptic fibers are myelinated, whether these fibers are large or small, the myelin sheath terminates abruptly a short distance proximal to the contact surface¹⁰ (see fig. 1, B, *m*). Furthermore, a differentiated myelin layer on the surfaces of the perikaryon, dendrites, or axon endings, has not as yet been detected by histological methods. This finding is in accord with the polarization optical studies of Chinn (1938), who found a thin lipid-protein sheath covering the dorsal root ganglion cells of the frog but no such layer over the perikaryon and axon hillock of the motor horn cells.

SPATIAL RELATIONS. Having considered the general aspects of structure in the region of the synapse, and the biological materials which may be involved in synaptic function, we come to a perhaps more formidable task. This involves dealing with the multiplicity of endings of axons in the nervous system, and their contiguous relations to the surfaces of other nerve cells. That this multiplicity of endings, with the accompanying complexities and diversities of interconnec-

²³ Bronk and Brink, 1939; Rosenblueth, 1940; Libet and Gerard, 1941.

²⁴ Arnold, 1863; Beale, 1863; Ehrlich, 1886; Retzius, 1889; Huber, 1899; Smirnow, 1900; Ramón y Cajal, 1906; Pitzorno, 1913, 1914.

tions, is of primary functional importance is axiomatic. Obviously, however, for purposes of analysis, one must reduce the complexities of construction and arrangement of neurons to one or another common denominator. In this instance the process will be that of limiting the discussion to morphological relations which might in any way affect the firing of single neuron units, since this is the ultimate problem of synaptic physiology. The discussion which follows will consist of two sections, the first dealing with monosynaptic systems, or neurons which might conceivably be excited by means of a single axon terminal, and the second dealing with the far more common polysynaptic systems, or neurons which must be excited by means of more than one axon terminal.

Monosynaptic Systems. A common postulate is that trans-synaptic excitation requires the disturbance of a given area of surface of a nerve cell by summation of activities of a number of closely spaced terminal membranes.²³ In the case of the small boutons this is suggested by the anatomical fact that these boutons are usually the terminations of collaterals of an arborizing axon, and that the fields of distribution of the boutons derived from two or more axons overlap. The arborization of a fine fiber near its termination could permit the excitation of several or many points on the surface of one or more cells of termination, and in the case of a particular cell of termination the ending of a single fiber by means of many boutons presumably would allow for disturbance of a larger surface area than could be effected by a single terminal knob.

The above assumption does not logically exclude the possibility of the existence of monosynaptic systems, that is, neurons receiving only one synaptic terminal. Nevertheless, McCulloch (1938) has questioned the justification for attributing the property of irreversible conduction to the individual synapse because of lack of evidence, first, for the existence of a monosynaptic system, and second, for effective excitation across a single synapse in either direction. This point of view fails to take into account considerable histological and physiological data of great interest. Johnson (1923) has described axo-axonal synapses of the segmental giant fibers in crustaceans which histologically are not unlike the large club-ending synapses in vertebrates.¹⁹ A membrane is present between the two elements of the synapse in *Cambarus*, although he describes partial fusion in other species. When the membrane is seen, there is a constant staining difference on the two sides, and experiments showed no passage of degeneration, or of methylene blue, across the membranous barrier. A similar situation is described in the segmental giant fibers of annelids by Stough (1926, 1930) and by Young (1936). The former offers evidence for, and reviews the literature on, polarized conduction of the segmental giant fibers, and suggests a significant correlation between this polarization and the differential staining of protoplasts on opposite sides of the synaptic septa, since the darker staining protoplasm is constantly present on the proximal side of the membrane in both medial and lateral giant fibers. These fibers, upon which depend the longitudinal contractions of the earthworm (Yolton, 1923), conduct

²³ Eccles and Sherrington, 1931; Lorente de N6, 1935, 1939; McCulloch, 1938; Young, 1939; and others.

in opposite directions as determined by isolated cutting of the fibers and stimulation of opposite ends of the body. Eccles, Granit and Young (1933), however, have found that impulses set up by induction shocks can pass in either direction along the giant fibers, presumably across the septa, and Young (1936) suggests that this reversible conduction across the membranes may be due to the similar dimensions of the membranes in contact. If this is true, and we are dealing with a monosynaptic system, it follows not only that reversibility is a property dependent on the relative dimensions of the contiguous synaptic membranes, but that in a monosynaptic system a single impulse may be effective across the synaptic barrier (see discussion of Lillie, 1936).

Young (1938, 1939) has shown in the stellate ganglion of the squid, in which the synapse involves a large contact surface, that a single impulse in the presynaptic fiber can set up a single impulse in the postsynaptic fiber. In this case the monosynaptic system involves nonsymmetrical synaptic surfaces, which, according to Young, may account for irreversibility at this synapse as compared with the symmetrical single synapses of the segmental giant fibers of the annelids.

Analogies in the vertebrate nervous system are not easily found, since most of the described neuronal systems are polysynaptic. However, the difficulty of demonstrating histologically the larger synaptic membranes in vertebrates may be partly responsible for this fact, smaller synaptic axon endings usually being associated with polysynaptic systems. The large club-endings on Mauthner's cell (fig. 2), first described by Bartelmez (1915) in *Ameiurus*, are here part of a polysynaptic system composed of many large and small endings on the dendrites, soma, and axon of Mauthner's cell (Bodian, 1937), but similar large club-endings on much smaller cells in the teleost tegmentum, described in the trout by Beccari (1934) and in the goldfish by Bodian (1937, 1940) present problems perhaps analogous to those of the monosynaptic systems in invertebrates since a single synapse involves a relatively large part of the cell surface (fig. 1, B). Such endings, which are transitional in form with cup-shaped endings, calyciform endings, and arborizing endings of large fibers in many centers in various vertebrates, have been described in the tangential nucleus of teleosts,²⁶ other tegmental cells of teleosts,²⁷ the trapezoid nucleus of mammals,²⁸ the oculomotor nuclei of teleosts,²⁹ the tangential nucleus of reptiles,³⁰ the ciliary ganglion of birds and of reptiles,³¹ and the retina of vertebrates.³²

Bodian (1940) has described the synaptic composition of some of the smaller cells in the tegmentum upon which single large club endings terminate (fig. 1, B). These endings have a diameter at the synaptotagma of about 7 to 9 μ , whereas the cells upon which they terminate, almost spherical in shape, have least and greatest diameters of about 14 to 16 μ . The surface of the soma of

²⁶ Ramón y Cajal, 1908, 1934; Tello, 1909; Beccari, 1931, 1934; Bodian, 1937, 1940.

²⁷ Beccari, 1934; Bodian, 1937, 1940.

²⁸ Held, 1897; Ramón y Cajal, 1934.

²⁹ Beccari, 1909, 1930; Bodian, 1940.

³⁰ Beccari, 1911; Segarra, 1926.

³¹ Carpenter, 1911; von Lenhossek, 1911, 1912.

³² Ramón y Cajal, 1932; Polyak, 1936, 1941.

such a cell, exclusive of dendrites, is roughly 500 sq. μ , whereas the surface of the club synaptotlemma is approximately 50 sq. μ . Other synaptic endings, derived from axons other than those forming the club endings, are present in these cells in moderate numbers but these are extremely small as compared with the club-ending, perhaps of the order $\frac{1}{2}$ to 1μ . In short, such a cell may have roughly $\frac{1}{10}$ of the surface of its body in contact with the club-ending alone, and presumably a single impulse might be enough to excite the cell, regardless of the activity of the smaller endings. These club-endings are apparently derived from vestibular root fibers, and might be expected to dominate the synaptic field of the terminal cell when active. Such a polysynaptic system, in that case, might become a functional monosynaptic system, in which a single impulse might excite across the synaptotlemma.

Polysynaptic Systems. A nerve cell, upon which two or more axons terminate, may be considered, along with the axonic terminals, as a polysynaptic system. Such systems represent the predominant types of synaptic aggregates associated with single neurons, especially in the vertebrate central nervous system, and probably represent the functional units with which one must deal in considering effective trans-synaptic excitation of such a single neuron. The synaptic composition of nerve cells varies greatly in different nerve centers, and to a lesser degree in any particular center, with respect to form, size, and numbers of synaptic endings, the distribution of these endings on the cell of termination (body, dendrite, axon), and the localization of different types of endings on specific regions of the synaptotlemma²² (see figs. 1 and 2). The analysis of the factors in synaptic transmission in such systems in terms of both temporal and spatial relationships, and of normal function, is a formidable task, but the fascination of the problems which can be approached with electrical methods applied to particular anatomical systems will no doubt ensure continued detailed studies of favorable systems.

Neurons with heterogeneous endings. Synaptic endings may be quite variable in numbers, sizes, and forms on nerve cells of different centers, and it is hard to imagine that this variety, with however a relatively constant pattern for the cells of each center, is not of functional significance. In addition to the simple types of endings of the bouton or club variety, each of which may predominate on any particular cell, more complex endings exist in many centers. Such are the basket types of endings, which may represent either multiple endings of the simple end-bulb type or combinations of club-like and bulb-like terminals derived from branches of a single fiber. This is clearly apparent in the case of the more complex endings on the cells of the oculomotor and trochlear nuclei in the goldfish (fig. 1, C and D). On these cells almost any assortment of end-bulbs, clubs, and even small calyciform endings may be seen, often derived from branches of a single axon. These endings were described by Baccari (1909, 1930), who found that cup-like endings on the cells of the oculomotor and trochlear nuclei of larval trout, derived from ascending fibers in the medial longitudinal fasciculus and in appearance much like the cup or calyciform end-

²² Lorente de N6, 1934, 1939; Bodian, 1937, 1940.

ings on the cells of the tangential nucleus,²⁶ enlarged and broke up during embryonic development into a complex of end-bulbs. A similar increase of complexity of axon terminal arborizations with age has been described by Nonidez (1941) in subendothelial venous receptors near the heart in dogs. The differentiation of terminating axons with respect to the degree of branching of the axon, the morphological variation of single terminating units, and the position on the cell of termination occupied by the axon terminals may not inconceivably be analogous in a limited way with the differentiation of peripheral axon terminals, especially those of the sensory systems, such as found in the skin. In this complex sensory organ, the bulk of present-day evidence, well summarized by Woolard, Weddell and Harpman (1940), favors the allocation of "specific sensory functions to morphologically characteristic nerve endings". These investigators furthermore find that with respect to cutaneous pain endings, "fibers heterogeneously distributed through a spectrum of sizes bear endings of a single morphological type". Similarly, it is the specific sensory ending which determines both the adequacy of a stimulus (threshold) and the duration of the sensory discharge (Adrian, 1930). It is also possible, however, that the morphological variation of the endings of a single axon on a single cell is of significance and greatest diameters of about 14 to 16 μ . The surface of the soma of a cell functions chiefly with respect to the size of the terminal synaptic membrane (Beccari, 1930), or of its shape (Young, 1939), and to the numbers and arrangement of the endings. The size of the endings, as well as other cytological differences, such as the local physical constitution of the membrane may, for example, be correlated with the existence of specifically different synaptic thresholds of excitation.

The termination of a large fiber on a single cell by means of branching and terminal bulb formation is characteristic only of the endings in certain centers, as in the oculomotor nucleus of some teleosts, and has the effect of increasing the synaptic surface of even a large fiber. It may be significant that the details of the pattern of ending of the terminal bulbs of such large fibers on the teleost oculomotor neurons differ however on each cell (fig. 1, C and D). In the simple club endings of the type found on Mauthner's cell and on other cells of the teleost tegmentum, the terminating fiber, which may have a diameter as great as 7 μ , may end abruptly with little or no terminal swelling. The variability of the form and size of the endings in the central nervous system thus appears to be dependent largely upon conditions near the cell of termination during development, and secondarily upon the size of the terminating axon. The place of origin of the fiber has perhaps the least influence in determining the nature of the terminal axonic apparatus, as is also suggested by the regeneration experiments of Langley and others.³⁴ Thus, although the cells of various centers may carry quite diverse types of synaptic apparatus, in the form of simple endfeet, simple clubs, calyciform endings (tangential nucleus), or complex endings of the type found in the teleostean oculomotor nucleus, the endings are usually constant in type on the cells of any particular gray center. Mauthner's cell, which

³⁴ Langley, 1898; Harrison, 1910; Boeke, 1917, 1935; Baron, 1934; De Castro, 1934.

is a spectacular instance of a polysynaptic system in which localization of functional groups of axon endings occurs on the synaptotlemma, is a striking exception to this rule, since several of these functional groups are characteristically and constantly differentiated with respect to size as well as form of endings.⁸ Some reciprocal overlap of the functional groups of endings occurs, but the outstanding feature is the localization of some of the functional groups at comparatively distant regions of the enormous cell surface (fig. 2). This should offer an opportunity for testing experimentally the functional equivalence or non-equivalence of different portions of the synaptotlemma (dendrites, body, axon hillock). This type of localization of endings of diverse types or origins on the synaptotlemma is also characteristic of the Purkinje cell of the cerebellum, and also of other large cells with constant spatial orientation, and contrasts sharply with the overlapping arrangement of endings of diverse origin but of homogeneous types on the synaptotlemma, described by Lorente de Nó as "partially shifted overlapping" (1934). A simpler version of heterogeneity of endings is found in the nucleus motorius tegmenti and in some of the vestibular centers of teleosts, on the cells of which one occasionally finds, in addition to the customary numerous endfeet, a large club ending²⁷ (fig. 1, B).

Neurons showing reciprocal overlap of homogeneous endings. When one examines most nerve cells one finds that the endings are relatively uniform in type. Furthermore, in preparations in which more or less complete preservation and staining of endings has been attained, these endings tend to be relatively constant in number, as well as in form and distribution, on cells of the same functional center.²⁸ Although in some preparations one finds cells with either no endings, one ending, or very few endings, this is usually an indication of incompleteness of preservation or staining, as can be seen by comparison with better preparations. Yet on some of the smallest nerve cells in many locations it has not been possible to demonstrate contact terminals of any kind, as in the substantia gelatinosa Rolandi. Whether this indicates a deficiency of the histological methods, due perhaps to the difficulty of adequately preserving extremely minute endings, or whether synaptic functions are mediated in these cases without benefit of contact terminals, is not known. The large cells of the "motor" type in the vertebrate tegmentum and cord are covered principally by terminals of the endfoot type, which are usually so numerous in favorable material that an accurate estimate of their numbers is impossible. Estimates on mammalian motoneurons run as high as 1200-1800 for the cell body alone,²⁸ and as high as several thousand on cortical pyramids (Lorente de Nó, 1933). When large numbers of endfeet or small club-endings are present on the perikaryon, the inter-distances between endings are rarely greater than the diameter of the endings themselves (Bodian, 1940). Hoff (1932) found that as many as 8 boutons per 100 μ^2 may occur on the anterior horn cell of the cat, but Barr (1939) has estimated that an average of 16 boutons per 100 μ^2 occur on cat motoneurons (38 per cent of the cell surface), and Minckler (1940) finds on

²⁸ Ramón y Cajal, 1934; Bodian, 1937; Minckler, 1940.

²⁹ Barr, 1939; Minckler, 1940.

human motoneurons as many as 23 terminals per 100 μ^2 , and 14.5 per 100 μ^2 on cells of the sensory column in the cord. It is important to emphasize these figures, since it has been frequently assumed that the amount of surface of a nerve cell covered by synaptic terminals is relatively small in proportion to the total surface of the cell. On the cells of the nucleus motorius tegmenti the endfeet have their greatest concentration on the cell body, and sometimes are seen to be more widely spaced on the proximal parts of the dendrites. However, in general, the endfeet are more satisfactorily demonstrated on the cell body than on the dendrites.

In Ramón y Cajal's comprehensive final survey of the anatomical literature on synapses (1934) one finds no mention of axo-axonic synapses, although descriptions of such synapses were available (Beccari, 1920) and have since been amply verified.³⁷ Such synapses, in the form of terminal endfeet, are frequently seen on the axon hillock of mammalian cells, and in the case of large axons as far out on the axon as the axon neck or even the beginning of the myelin sheath. When present on the proximal part of axons, the endings are usually more widely spaced than on the perikaryon. In the case of Mauthner's cell at least the site of origin of the parent fibers of the endfeet on the axon hillock and axon neck is different from that of those fibers which terminate on other parts of the cell. In the functional interpretation of the presence of endfeet on axons of cells the possibility must be considered that impulses which impinge on the axon may have the same effect as artificial antidromic stimuli. Coghill (1934) has suggested that endings in this location may have an inhibitory function, and this hypothesis has been elaborated in greater physiological detail by Gesell (1940), and by Gerard (1941a).

The endfeet, in addition to being closely spaced on the tegmental cells, are rather evenly distributed, as can best be seen in tangential sections of the cell surface. The appearance of the smallest endfeet on many of the tegmental cells, with respect to their size, form, staining qualities, and distribution on the cell membrane, closely resembles that of the dendritic "thorns" of the cerebellar Purkinje cells (Bodian, 1940) which were first interpreted as endfeet by Held (1897).

The heterogeneous synaptic systems illustrate the morphological difficulties encountered in any assumption as to the functional equivalence of all types of synaptic endings. For experimental purposes, however, such an assumption may be valid for certain homogeneous polysynaptic systems, that is, neurons bearing endings of one morphological type. Such systems have been analysed in detail by Lorente de Nó,³⁸ and appear to be characteristic of mammalian motoneurons and interneurons. It has been known since early times that at least in many systems of endings of homogeneous sizes and forms, an overlapping distribution of endings derived from arborizations of two or more parent fibers occurs. This reciprocal overlapping described in some detail by Lorente de Nó (1934) is of considerable significance in any interpretation of synaptic function,

³⁷ Coghill, 1934; Bodian, 1937, 1940; Young, 1939; Barr, 1939.

³⁸ Lorente de Nó, 1933, 1934, 1935, 1938, 1938a, 1939.

and it is highly likely that it is a general phenomenon as regards synaptic systems consisting of numerous homogeneous endings. Whether overlapping or coextensive aggregates of endings of different fibers on a single cell may influence each other mutually (summation, inhibition) is problematical.³⁹ Analogous overlappings of coextensive homogeneous nerve endings in the cornea apparently have no such mutual influences (Tower, 1940; see also Weddell, 1941, 1941a) but this is perhaps not a fair comparison. Whether the caliber of the arborizing telodendria of a single terminating axon, the size of the boutons, and their spacing on the cell of termination affects the timing of the components of a single impulse as dispersed by the terminal arborization of the axon, is also conjectural.

AUTONOMIC GANGLIA. The synaptic structure of autonomic ganglia is of special importance because of the ready availability of these centers for experimental investigation. Unfortunately, although the incident impulses to the ganglion as a whole may be fairly homogeneous, the actual synaptic composition in relation to any particular cell may be complex. Anatomically there is no evidence for any fundamental difference between sympathetic ganglion and central nuclei with respect to the structure and arrangements of synaptic systems on single cells or cell groups.

The endings on sympathetic ganglion cells were first clearly described in 1886 by Ehrlich, who noted the spiral course of some of the incoming fibers⁴⁰ before they arborized around the cells of the ganglion and terminated by means of small terminal swellings. These findings have been essentially corroborated by many workers since that time (see Huber, 1899) and additional important details added (Lawrentjew, 1924). Huber noted that several fibers may contribute endings to a single cell in mammalian ganglia and this has often been confirmed. Likewise, a single entering fiber may contribute endings to several cells in the ganglion (Smirnow, 1900). The presence of collaterals of axons of sympathetic ganglion cells has often been described,⁴¹ and Lawrentjew has confirmed this finding by degeneration experiments (1924). He has also described arborizations of such collaterals which may contribute a group of several endswellings to as many as four neighboring nerve cells (1914). Such findings suggest the possibility of the existence of "self-re-exciting chains" of neurons, minus interneurons, in sympathetic ganglia, which could perhaps partly account for the prolonged excitatory state described in such ganglia following multiple preganglionic volleys.⁴²

In addition to the spiral and non-spiral entering fibers, and recurrent collateral fibers, all of which terminate by means of a few or many terminal swellings, often localized to one pole of the cell, Lawrentjew (1924), confirming Ramón

³⁹ See interesting evidence of spatial overlap and spatial summation in crayfish (Prosser, 1940).

⁴⁰ Spiral fibers associated with sympathetic ganglion cells were seen as early as 1863 by Beale and by Arnold.

⁴¹ Ramón y Cajal, 1893; von Lenhossek, 1894; Dogiel, 1899; Smirnow, 1900; Lawrentjew, 1914, 1924.

⁴² Bronk, Tower, Solandt, and Larrabee, 1938.

y Cajal (1906), has described fine incoming fibers which course along the dendrites, much like the climbing fibers of the cerebellum, and which possess collateral thickenings which form contact terminals on the dendrites. The origin of these fibers is unknown. Still other types of endings, of the club and basket type, perhaps of special functional significance, have been described in the ciliary ganglia of birds and reptiles,³¹ which could perhaps be studied experimentally.

It is obvious that peripheral synaptic ganglia, which are not only convenient for functional studies, but are also anatomically comparable with central synaptic areas, are strategic areas for further analyses of synaptic physiology.

RÉSUMÉ

The large numbers of endings on most nerve cells appear to have but one logical significance, namely, that most of them at any one time are concerned with maintaining a subliminal state of excitability,⁴³ which may or may not reach the threshold level of somatic potential, depending (perhaps, in some cases, predominantly (Tower, 1937)) on the occurrence of effective discharges from the functional center dominating the phasic activity at any particular time. This functional anatomical concept, and that of self re-exciting chains of neurons and collaterals, are corollaries of the general concept of functional cell-chain systems, and fit in well with the existence of morphological heterogeneity and overlapping distribution of endings on neurons of termination. How far, and in precisely what manner, the functioning of such systems can be influenced by more diffusely acting forces, such as chemical substances or potential fields,⁴⁴ remains to be seen.

Furthermore, the temptation and necessity to create diagrammatic generalizations should not be allowed to obscure a fact which is most relevant for experimental investigation, namely, the great anatomical variability of particular synaptic systems. It is with particular systems that the experimenter must deal, and in the interpretation of data obtained from particular systems generalized diagrams are often misleading. The understanding of the specific characteristics of the anatomical set-up being used in particular physiological experiments is essential for the interpretation of the results, but, as often as not, physiological experiments reveal the fact that we are still ignorant of many relevant anatomical relationships.

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⁴³ Lorente de Nó, 1934, 1939.

⁴⁴ Gerard, 1941, 1941a; Libet and Gerard, 1941.

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ORGANIC CHEMICAL INDUSTRIAL HAZARDS TO HEALTH

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A survey of industrial hazards to health caused by organic chemicals, as presented in the following pages, has necessarily to be restricted to the essential compounds; and for detailed information, whenever possible, references will be given to reviews on the various topics. For this reason the discussion will deal with chemical groups rather than with individual compounds, and, in each of these, their industrial use, the general character of their toxic action, and the general trend of the toxic properties within such groups will be analyzed. The arrangement according to chemical groups instead of in respect to toxicologic effects, such as irritants, blood poisons, circulatory poisons, nerve poisons, etc., appears to be advantageous because such classification avoids overlapping and will give a better basis for the appraisal and further study of such toxic substances as may gain importance from the industrial point of view. For this reason the subject will be arranged according to three main groups: the aliphatic hydrocarbons, the aromatic hydrocarbons, and the naphthalene derivatives; and in each the compounds produced by the introduction of certain radicals, such as hydroxy-, amino-, nitro-, and others, will be discussed.

A. THE ALIPHATIC HYDROCARBONS AND THEIR DERIVATIVES. 1. The *aliphatic hydrocarbons* may be subdivided into the following groups: Paraffins, olefines, diolefines, acetylenes, cycloparaffins and unsaturated cycloparaffins. The lower members of these groups with 1 to 4 carbons are gases at ordinary temperature; those with 5 to 15 in the paraffin, to 18 in the olefine, and to 6 in the ring of the cycloparaffin series are liquids; and those of higher carbon content are solids. The liquid aliphatic hydrocarbons are constituents of petrol ether, benzine and coal oil. But the composition of these solvents is not uniform and the toxicological properties of different brands of benzine differ quantitatively and qualitatively, especially if contaminated with aromatic hydrocarbons. Some of these hydrocarbons, such as cyclohexane and its homologues, are used in pure form; and others, such as benzine and coal oil, are used in mixture. They are used extensively as solvents in the chemical, lacquer and related industries.

The *paraffins* of the general formula C_nH_{2n+2} have narcotic properties which increase from hydrocarbons with one carbon to hydrocarbons with eight carbons in the chain. It appears that with some members the margin of safety between narcotic and toxic concentration is very small, and that hydrocarbons with straight chains are more toxic than those with branched chains containing an equal number of carbons. The liquid members of this group cause irritation of the nerve endings in skin and mucous membranes, increasing in intensity up to hydrocarbons with seven carbon atoms.

Similarly, the narcotic and toxic actions of *olefines* of the general formula C_nH_{2n} increase with the molecular weight and in inverse ratio to their solubility in water and in direct ratio to their solubility in oil. In opposition to the be-

havior of the corresponding paraffins, olefines with more than four carbons in the chain cause increased reflex excitability, and those with more than three carbons are said to affect the circulation also, partly by stimulation of the vagus. It appears, further, that some of these compounds may cause injury of the liver, whereas the irritant effect appears to be slightly less marked than with the saturated hydrocarbons.

Introduction of a second double bond yields *diolefines* and increases the narcotic action, and the same holds true for the introduction of a triple bond, as in acetylene.

The *cycloparaffins*, which represent ring structures containing from three to six carbons, have narcotic properties which increase with the number of carbons within the ring system. With introduction of side chains into these ring systems the toxicity increases with the length of the side chain, which increase is especially marked in the cyclo-hexane series between the methyl and ethyl radicals. As observed with the olefines, introduction of a double bond increases the narcotic and toxic action, and the margin of safety between them appears to be quite small. Like other hydrocarbons, cycloparaffins may cause irritation of the skin and mucous membranes.

In comparison with the aromatic hydrocarbons, the effect of aliphatic hydrocarbons on the blood and blood-forming organs, their fate in the metabolism, and their excretion, has not been adequately studied, and it appears that the saturated and unsaturated cycloparaffins especially may prove interesting in this respect.

2. The *aliphatic alcohols* are formed by the introduction of one hydroxy group into aliphatic hydrocarbons, and this tends to increase the narcotic action, at least with the lower homologues of this series. Only the lower members of this series, from methyl to amyl alcohol, are used as industrial solvents; those with more than five carbons are slightly volatile, and those containing ten carbons and more are solids at normal temperature. With those members of the series which are of industrial interest, both the boiling point and the partition coefficient $\frac{\text{oil}}{\text{water}}$ increase with the molecular weight, and these physico-chemical characteristics are presumably largely responsible for the duration and intensity of their narcotic action.

They are readily absorbed from the mucous membranes of the respiratory and gastro-intestinal tracts, whereas their absorption through the intact skin appears to be limited.

In regard to their fate in the organism, the primary alcohols of the type $R-OH$ are oxidized to aldehydes and monocarboxylic acids, whereas secondary alcohols of the type $R-OH-R$ are oxidized to ketones and carboxylic acids. Methyl and ethyl alcohols are representatives of the former type, and isopropyl alcohol is a representative of the latter type.

As pointed out before, the narcotic action of alcohols increases with the molecular weight, and it is paralleled by an increase of the boiling point and the partition coefficient $\frac{\text{oil}}{\text{water}}$. Alcohols with branched chain (iso) appear to be

less effective than those with straight carbon chains. Whether this should be explained on the basis of physico-chemical properties, or whether it is associated with a different speed of oxidation appears to require further study. In some instances, as is the case with butyl alcohols, the secondary alcohols are more effective than the normal compounds, presumably on account of their greater vapor tension.

Toxicity and narcotic action do not run parallel. The lowest member of this group with the least marked narcotic action, methyl alcohol, is more toxic than the higher homologues, and it appears that the toxicity is in part determined by the metabolites, that is, in this specific case, by the formation of formaldehyde and formic acid. In addition, the toxicity is partly determined by the speed of the oxidation of the alcohols in the organism, partly, as far as the excretion through the lungs is concerned, by their volatility, and partly by the speed of their urinary excretion.

The local irritant action of alcohols depends in part upon their efficiency as fat solvents, in that they remove in this way the protective fat layer of the skin and thus render it more susceptible to thermic and atmospheric influences. With the lower homologues, which have a comparatively high affinity to water, dehydrating effects may also be involved. The precipitant action of alcohols on proteins, which increases from methyl to amyl alcohol and which is probably related to corresponding changes of the surface tension, seems to be another factor in the irritant action. Especially with the higher members of this group the local irritation is followed by analgesia which is especially marked with butyl and amyl alcohol and which may be connected with the above effect.

In regard to the possibility of habituation to alcohol, this depends within certain limits upon the individual constitution, especially on the metabolic rate and on the functioning of the liver which determines the speed of the destruction of the toxic agent.

Of *bivalent alcohols* the lower homologues, especially ethylene glycol, $\text{HOCH}-\text{CH OH}$, and its derivatives, are extensively used as solvents for cellulose lacquers. The introduction of a second hydroxy group reduces the narcotic action as compared with that of monovalent alcohols. Ethylene glycol itself has very moderate narcotic and some irritant properties, and, on account of its high boiling point, systemic effects from the inhalation of its vapors have not been observed. With oral administration, however, it will cause severe injury of the kidney which is, in part, due to its fate in the metabolism, resulting in the formation of oxalic acid. This appears to depend on the existence of two carbon compounds, both carbons of which are partially oxidized. Among the ethers of ethylene glycol, diethylene glycol has moderate irritant and narcotic properties, and, when taken by mouth, marked nephrotoxic action which is more marked with the double ether of ethylene glycol, dioxan. On account of its low boiling point the latter may also be dangerous by absorption through the lungs. With the monoalkyl ethers of ethylene glycol, the nephrotoxic action and the irritant action appear to increase from the methyl to the butyl derivative. The narcotic action is not very marked and its trend within the series has not yet been estab-

lished. The diethyl ether is slightly less active than monoethyl glycol. Of the esters, mono- and diacetyl glycol have a distinct nephrotoxic action, being at least partly hydrolysed and oxidised to oxalic acid. Of the mixed ether-esters, methyl glycol acetate has a more marked nephrotoxic action than the ethyl ether, the narcotic and irritant properties being not very marked.

In opposition to ethylene glycol, propylene glycol is comparatively little toxic although the irritant action, especially on mucous membranes, appears somewhat more marked, and its monomethyl ether is said to have only moderate irritant, nephrotoxic and narcotic properties. The toxicity is further reduced with the next higher homologue, butylene glycol, which is nonirritant and of low narcotic and nephrotoxic action, which properties are only moderately increased by esterification with acetic acid.

Of *trivalent alcohols*, only glycerol is of industrial importance. It is largely used as a softening agent. It has some irritant and moderate narcotic and nephrotoxic action, the latter becoming manifest only after the ingestion of fairly large doses. Under certain conditions it may be utilized by the organism and changed to glucose, which effect becomes more marked with the higher homologues, mannite and dulcitol which, however, are at present of no great industrial importance.

3. Oxidation of primary alcohols yields *aldehydes*, of which formaldehyde and acetaldehyde are the main representatives. They have narcotic and irritant properties, the former being prevalent with the higher and the latter with the lower homologues, depending partly upon their solubility in water, which is greater with the lower members of this group. The lowest member of this series is mostly used as a disinfectant and in the manufacturing of artificial resins, and its polymer, paraldehyde, is used industrially in mixture with alcohol as a solvent for nitrocellulose. As compared with the monomer, the irritant action of the polymer is considerably reduced, whereas the narcotic action is materially increased, so that it can be used effectively as a sedative and soporific. The irritant action is considerably increased by the introduction of a double bond as in acrolein which is formed by dehydration of glycerol as by heating fat and oils. It has very marked irritant properties, whereas its narcotic action is negligible. Of cyclic aldehydes, only furfural (furfural) is of industrial importance, being used as solvent for cellulose esters and waxes, resins and caoutchouc, and also in the vulcanization of rubber. It has irritant properties, but on account of its high boiling point the stimulant and depressant effect on the central nervous system is of no industrial toxicological importance.

Condensation of aldehydes with monovalent alcohols yields *acetals*. Of these, only the condensation product of acetaldehyde with ethyl alcohol, diethylacetal, has any industrial importance, being used in the paint and varnish industry, but it can hardly be considered an industrial poison and, although moderately irritant, it has been used therapeutically on account of its narcotic action.

4. Oxidation of secondary alcohols yields *ketones*. These are characterized by a $C=O$ group bound to two alkyl radicals. As in the case of aldehydes, the oxygen is connected with the carbon atom by a double bond, which accounts

for the great activity of these compounds. They are used extensively as solvents for celluloid, cellulose acetate, and nitrocellulose in the lacquer industry. Their vapors cause more or less marked irritation of the mucous membranes, which is, however, less pronounced than with aldehydes, and they have narcotic properties which are more marked than those of the corresponding alcohols. With the simple ketones, such as dimethyl ketone (acetone), methyl-ethyl ketone (butanone), methyl-propyl ketone (propanone-2), methyl-n-butyl ketone (hexanone-2), methyl-n-amyl ketone (heptanone-2), and methyl-n-hexyl ketone (octanone-2), the narcotic action increases with the number of carbons, and the degree of depression is closely paralleled by an increase of their partition coefficient, $\frac{\text{oil}}{\text{water}}$, and of their surface tension. A comparison of the narcotic action of hexanone-2 with that of other ketones with six carbons but of different structure, such as methyl-iso-butyl ketone (hexone), methyl-iso-butyl ketone (mesityl oxide), diacetyl ethane, cyclo-hexanone and methyl cyclohexanone, indicates that those with a branched side chain (hexone) are less effective than those with a straight side chain (hexanone). Comparison of the narcotic action of cyclohexanone and methyl cyclo-hexanone indicates that the narcotic action is increased by the introduction of an aliphatic radical, as is also the case with the introduction of a double bond (mesityloxide). The presence of a second ketone group, as in diacetyl ethane, reduces the narcotic action.

5. Upon further oxidation, primary alcohols yield *monobasic acids* of the general formula RCOOH . The lower homologues of this group, such as formic and acetic acid, are used as mordants in the textile industry. They have no narcotic properties, but their vapors produce irritation of the mucous membranes.

Oxidation of bivalent alcohols yields *dibasic acids* of the general formula HOOC R—R COOH . Of these only oxalic acid is of greater industrial importance, being extensively used as a bleaching agent. Locally it acts as an irritant, and following its absorption it has marked systemic effects which are closely affiliated with its ability to form insoluble calcium salts.

6. Complete oxidation of aliphatic hydrocarbons and of all carbonaceous material yields *carbon dioxide*. This is extensively used as a refrigerant. When solid, its contact with the skin may give rise to burns of various degree, whereas inhalation of the gas causes stimulation and, in high concentrations, paralysis of the respiratory center.

Less complete oxidation yields carbon monoxide. This is a by-product of many industrial processes. Its toxic action is mainly based on its great affinity to hemoglobin, thus preventing the formation of oxyhemoglobin and leading mainly to anoxemia, the sequelae of which vary with the intensity and duration of exposure. Among other aliphatic oxides, *ethylene oxide* should be mentioned. It is used as a fumigant and intermediate in chemical industries. It has narcotic and irritant properties.

7. Condensation of aliphatic acids with alcohol yields *esters*. These are used extensively as solvents in the lacquer and other industries. The lower members of this series and, less readily, the higher homologues are hydrolyzed with the

formation of their constituents, and their toxicity depends partly on their split products. The dangers from their absorption through the lungs and the skin are determined partly by their solubility in water and partly by their volatility.

The formic acid esters, methyl, ethyl, n-butyl and iso-amyl formate, are especially easily hydrolyzed and are, therefore, generally more toxic than the esters of higher aliphatic acids, and their irritant and narcotic action increases from methyl to iso-amyl formate, whereas cyclic formates are said to be less toxic, approaching, in this respect, amyl acetate.

The acetic acid esters, methyl, ethyl, propyl, n-butyl and iso-amyl acetate, have irritant and narcotic properties which increase, with the molecular weight, from the methyl to the amyl compound. Of the cyclic acetic acid esters, cyclohexylacetate is said to be three times more toxic, but three to five times less volatile, than amyl acetate, whereas benzyl acetate is said to be more irritant.

Esters of propionic and butyric acid, although also used as solvents, have evidently not been studied toxicologically, and esters of higher aliphatic acids, such as ethyl and butyl lactate, are said to be little toxic.

In man, exposure to the vapors of esters has been found to cause irritation of the mucous membranes (conjunctivitis and irritation of the upper respiratory tract), their contact with the skin may lead to irritation and secondary infections, and, with some, systemic effects of various natures have also been reported.

8. Condensation of two molecules of alcohol yields *ethers* of the general formula $R-O-R$ which have marked narcotic properties. Of the three lowest members of this group, dimethyl, diethyl and dipropyl ether, only the diethyl ether is of great industrial importance, being used as a solvent in the manufacturing of smokeless powder and artificial silk (Chardonnet process).

9. Introduction of one or more halogen atoms into aliphatic hydrocarbons yields a great series of halogenated hydrocarbons which are extensively used in industries. Substitution of one, two, three, or four hydrogen atoms of methane by chlorine yields *mono, di, tri and tetra-chloro methane*, the two latter being better known as chloroform and carbon tetrachloride. The lowest member of this group is used as a refrigerant and the others play a very important rôle as solvents, especially for fats and oils. The hemolytic, antiseptic and narcotic properties of these compounds increase with their molecular weight and in inverse ratio to their solubility in water. The irritant action on the mucous membranes is much more marked with the lower than with the higher members, and the injurious effect on the liver increases with the number of chlorine atoms. Substitution of bromine for chlorine decreases, as a rule, the narcotic action but increases the toxicity, and the same holds true, as far as is known, to a larger extent for iodine derivatives. On the other hand, the substitution in carbon tetrachloride of one or two chlorine atoms by fluorine, resulting in the formation of monofluorotrichloromethane and difluoro-dichloromethane, both of which are used as refrigerants, reduces the toxicity considerably. The lowest member of this series yields, upon hydrolysis, methyl alcohol, and this explains, in part, the toxicological picture.

Introduction of one or more chlorine atoms into the ethane molecule yields

monochlorethane, dichlorethane, ethylidenechloride, alpha-trichlorethane, beta-tri-chlorethane, tetrachlorethane, penta-chlorethane and hexachlorethane. These are extensively used as solvents in various industries. The antiseptic, narcotic and toxic action increases with the number of chlorine atoms introduced, and comparison of the narcotic action and the toxicity of isomers indicates that this is reduced by an unequal distribution of the chlorine atoms between the two carbon atoms. As in the previous series, replacement in tetrachlorethane of two chlorine atoms by fluorine reduces the toxicity materially, and replacement of the chlorine atom by bromine and iodine, as in ethyl chloride, ethyl bromide and ethyl iodide, decreases the narcotic action and increases the toxicity.

Introduction of one or more chlorine atoms into ethylene yields *vinylchloride, dichlorethylene, trichlorethylene and tetrachlorethylene.* These are also used very extensively as solvents in various industries. As compared with the ethane series, the toxicity of these compounds, especially in regard to the liver, is very materially attenuated, and their narcotic action increases with the number of chlorine atoms in the molecule.

Of chlorinated propane derivatives, *propylchloride and trichloropropane* are of industrial importance. These appear to be less toxic, both regarding the effect on the liver and the narcotic action, than the corresponding ethane derivatives.

Chlorine derivatives of butane, pentane and their higher homologues are at present of minor industrial importance, and very little is known in regard to their toxicology. Of chlorine derivatives of higher hydrocarbons, only allyl chloride and 2-chloro-butadiene have been studied more closely. Allyl chloride is considered to be one of the most toxic members of this group, having a very strong irritating action, especially on the lungs. Its narcotic action is very weak and its injurious effect on the liver is apparently less severe. The chlorine derivative of butadiene, 2-chlorobutadiene (chloroprene), is the starting material for the synthetic rubber "Neoprene". It causes some irritation, has narcotic properties, and, with sufficiently high doses and prolonged exposure, will cause injury of liver and kidneys.

Introduction of chlorine into alcohol molecules yields chlorinated alcohols. Of these, *ethylene chlorhydrine*, $\text{CH}_2\text{Cl}-\text{CH}_2\text{OH}$, and *dichlorhydrine*, $\text{CH}_2\text{Cl}-\text{CHOH}-\text{CH}_2\text{Cl}$, are used as solvents. They cause irritation of the mucous membranes, the former causing, in addition, nervous injuries and fatty degeneration of the liver, heart and kidneys, and the latter affecting also the circulation. Trichlorisobutyl alcohol and tribromooethyl alcohol (avertin) have marked narcotic properties but are not important from the industrial point of view.

Of the halogenated aldehydes, only *chloralhydrate* should be mentioned. This has marked sedative and hypnotic properties being, however, easily injurious to the heart.

Of the halogenated ketones, *chloroacetone, bromoacetone, brom-methyl-ethylketone, dibrom-methyl-ethylketone and iodo-acetone* should be mentioned. They are, at present, of little industrial importance but have been used, on account of their very marked irritant action, especially on the eyes, in chemical gas warfare.

Of the chlorinated esters, *methyl sulfuryl chloride and ethyl sulfuryl chloride* are

used in the chemical industries. They cause severe irritation of the eyes and the respiratory tract and, in addition, systemic effects. Others, as *methyl-chloro-formate*, *chlormethyl-chloro-formate*, *dichlormethyl-chloro-formate*, *trichlormethyl-chloro-formate*, *methyl-bromoacetate* and *ethyl-iodoacetate*, cause severe irritation of all mucous membranes and have been used, for this reason, in chemical gas warfare.

Of chlorinated ethers, only β, β' -*dichlorethyl ether*, $C_2H_4Cl-O-C_2H_4Cl$, is of some industrial importance, being used as solvent in the textile industries. It has marked irritant and some narcotic properties, being also injurious to liver and kidneys.

10. Introduction of an amino group into aliphatic hydrocarbons yields *aliphatic amines*, and, depending upon the number of alkyl radicals introduced into the ammonia molecule, mono-, di- and tri-alkyl amines may be distinguished. As an increasing number of alkyl radicals is introduced the original character of ammonia is attenuated. There exists little information regarding their toxicologic action, and they are, at present, of limited industrial importance.

The same holds true for *amino alcohols* of the type $NH_2 \cdot R \cdot OH$. Of these, triethanolamine, $N(C_2H_4OH)_3$, is being used as solvent and detergent. On account of its high boiling point it offers very little risk in regard to toxic effects from inhalation, but absorption through the skin appears to be possible, and this may result in injury of liver and kidneys.

11. Introduction of one or more nitro groups into aliphatic hydrocarbons yields *nitroparaffins*. The lower members of this series, *mono-nitro-methane*, *-ethane*, *-propane* and *-butane*, cause local irritation and injury of the liver, these effects increasing with the molecular weight. *Tri-nitro-methane* may be formed as a by-product during nitration of toluene. It causes marked irritation and may lead to formation of methemoglobin. Its chlorine derivative, *chloropicrine*, is used in fumigation and has marked irritant properties. Of other chlorinated nitroparaffins, *1-chloro-1-nitromethane* and *2-chloro-2-nitropropane* have marked irritant properties but are evidently not more injurious to the liver than the corresponding, unsubstituted, nitro compounds.

Introduction of a nitro group into alcohols, as in *2-nitro-2-methyl-1-propanol*, *2-nitro-2-methyl-1,3-propanediol* and *2-nitro-2-methylol-1,3-propanediol*, results in irritant materials, and, with the latter, other toxic effects may be attributed to the formation of methyl alcohol in the organism.

12. Esters of aliphatic alcohols with nitrous acid of the general formula $R-ONO$ are called alkyl nitrites. Of these, *ethyl nitrite* is used in the chemical industry and may be formed in the manufacturing of mercury fulminate. It produces vasodilatation and fall of blood pressure and may cause methemoglobin formation. The higher homologue, *isoamyl nitrite*, is used in chemical industries and as a therapeutic agent on account of its vasodilator and pressor action; in larger doses it is liable to cause central stimulation and methemoglobin formation.

Representatives of *aliphatic esters of nitric acid* are *tri-nitroglycerol* and *di-nitro ethylene glycol*. These are extensively used in the explosives industry. Their effect closely resembles that of alkyl nitrites because they are partly hydrolyzed

and reduced in the organism with the formation of nitrites. They cause vasodilatation, fall of blood pressure and, in large doses, central stimulation and methemoglobin formation. Nitro derivatives of higher polyvalent alcohols, such as *erythritol tetranitrate*, have a similar but somewhat delayed action.

13. Aliphatic *nitriles* are characterized by the group $\text{—C}\equiv\text{N}$. The lowest member of this series is formyl nitrile or *hydrocyanic acid*. This is used as a fumigant and may be encountered in the mining, chemical and other industries. It paralyzes the cellular respiration, and, in sufficiently high concentrations, may be rapidly fatal. The corresponding compound in the dibasic series is dicyan. This is more irritant but less toxic than hydrocyanic acid. The higher homologues, such as acetonitrile and propionitrile resemble, qualitatively, hydrocyanic acid but are less toxic. The halogene derivatives of hydrocyanic acid, cyanchloride and cyanbromide, have marked irritant properties, and, in addition, have a depressant effect on the cellular respiration similar to that of hydrocyanic acid. They are used with other fumigants as warning agents and may be encountered in chemical industries.

Isonitriles of the general formula $\text{C}\equiv\text{NH}$ are used in the chemical industry, and their action is similar to that of the nitriles.

Thiocyanates or rhodanates of the general formula RSCN are used as insecticides. The lower homologues split off hydrocyanic acid but become more stable with increasing molecular weight, so that with dodecyl and lauryl sulfo-cyanate this effect is absent. However, with sufficiently high doses and with sufficient duration of exposure, the latter may cause degenerative changes of the liver and of other organs.

14. Regarding *aliphatic sulfur derivatives*, carbon disulfide is, by far, the most important, being used as a fumigant and also in the rubber, rayon and various chemical industries. In large doses it acts as a narcotic agent, whereas in lower concentrations it acts mainly as a nerve poison for the peripheral and central nervous system, leading to peripheral neuritis and psychic disturbances.

Replacement of the oxygen atom in alcohols by sulfur leads to *mercaptanes* of the general formula RSH . They are stenchers and are used as intermediates in chemical industries. Ethyl mercaptane causes primary stimulation and, later, paralysis of the respiration similar to that produced by hydrogen sulfide. Chlorinated mercaptane, perchlormercaptane, $\text{CCl}_3\text{—SCl}$, has marked irritant properties.

Sulfurated ethers of the type RSR may be encountered in the chemical industries. They may cause paralysis of the central nervous system. Of great practical importance is the chlorine derivative, dichlorodiethylsulfide, mustard gas, which is used in chemical industries and in gas warfare. It has marked irritant properties, causes vesication, and acts as a cell and nerve poison.

B. THE AROMATIC HYDROCARBONS AND THEIR DERIVATIVES. 1. The *aromatic hydrocarbons*, the lowest homologues of which are benzene, toluene and xylene, are used extensively as solvents in lacquers and spray paints and as starting materials in the manufacture of dyes and in the explosives industries. In judging the toxicity of these materials one has to distinguish between acute and

chronic toxicity and the former may vary according to the form of absorption. The aromatic hydrocarbons generally produce a progressive depression of the central nervous system which, especially with toluene, may later on be associated with convulsions. The reflex excitability usually persists until shortly before death which results from respiratory paralysis. The narcotic and irritant action of toluene and xylene is greater than that of benzene, whereas with continued exposure to lower concentrations the latter is by far the more dangerous on account of its hematotoxic action. In addition to the effect on the central nervous system they cause peripheral vasodilatation. Benzene and those of its derivatives with substitution of two hydrogen atoms in para position (p-xylene, p-methyl-ethyl benzene, p-methyl-propyl benzene and p-diethyl benzene) cause tremors of the body. These gradually pass into twitchings which may persist until the beginning of the paralytic stage. Hydrocarbons with a branched side chain (isopropyl benzene) are generally less toxic than those with a straight side chain (propyl benzene) and the introduction of two or three groups usually produces less toxic substances than that of a single group with an equal number of hydrocarbons. Whereas the acute toxicity of aromatic hydrocarbons has been studied quite extensively, the effect of continued exposure of the higher homologues, especially on the blood picture and on the skin, needs further study. The available information indicates that the hematotoxic action of benzene, resulting in leucopenia and anemia, is considerably attenuated by substitution of hydrogen atoms, as in toluene and xylene. The reason for the outstanding hematotoxic action of benzene, as compared with its homologues, is probably its different fate in the organism, in that it is oxidized in the organism to phenol, pyrocatechol and hydroquinone, whereas the higher homologues are usually oxidized in the side chain with the formation of carboxylic acids. The intensity of the oxidation and the amount of hydroxy derivatives, especially of polyphenols, formed in the liver, may vary with the metabolism. This may explain the variations in susceptibility of different individuals. Such studies may promote considerably the understanding of the mechanism of the toxic action of benzene and its homologues.

2. Introduction of one hydroxy group into the benzene ring with the formation of phenol increases the convulsant and decreases the narcotic actions. Phenol is used extensively as an antiseptic and as a starting material in the chemical industry, and it forms one constituent of phenol-formaldehyde plastics. The ingestion of sufficiently large doses of phenol may lead to corrosion of the upper digestive tract and result in convulsions of the spinal type and collapse. Continued exposure to lower concentrations may lead to nervous and nutritional disturbances and injury to the kidney. Contact with the skin will cause irritation and, when continued for a sufficient length of time, may cause necrosis.

As in the case of olefinic hydrocarbons the toxicity of phenol is decreased by hydrogenation, hexahydrophenol (cyclohexanol) being less toxic than phenol. The same holds true for the higher homologue (methyl cyclo-hexanol) which, as well as the former, is used as a solvent for fats, oils, waxes, rubber, etc.

The toxicity of phenol is also reduced by closing the hydroxy group by etheri-

fication with alkyl groups. The methyl ether of phenol (anisol) and its ethyl ether (phenetol), both of which are used in the chemical and explosives industries, are considerably less toxic than phenol and do not cause convulsions.

A similar effect is produced by the introduction of alkyl groups into the ring of phenol. Introduction of one methyl group yields cresol (methyl-hydroxy benzene) and, depending upon the position of these two groups to each other, ortho, meta and para cresol may be formed. These are used as antiseptics and wood preservatives, and also in the chemical industries. They are less toxic than phenol in respect to their systemic action, although their local action is of the same order. They all depress the central nervous system and differ among themselves in that the ortho compound is generally the most toxic and the meta compound the least toxic, the para compound being intermediate. It has been stated that continued exposure to vapors of cresol may cause changes of the blood picture which are similar to but much less severe than those observed with benzene.

Among the esters of phenol and its homologues, the triphenyl and tri-ortho-cresyl phosphoric acid esters are used as plasticizers and solvents for various resins and nitrocellulose. Whereas the former is not very toxic, the ingestion of tri-ortho-cresyl phosphate may lead to peripheral neuritis. The inhalation of its vapors may cause some irritation but nervous injury from this form of administration has not been reported.

Halogen derivatives of phenol are considered to be less toxic than phenol in regard to their convulsant action, but they may cause more or less severe irritation of the skin. Pentachlorophenol is of industrial importance, being used as a preservative for wood. Although administration of sufficiently high doses may cause toxic symptoms, such as accelerated respiration, fever, hyperglycemia, glucosuria, motor weakness and collapse, and the possibility of toxic effects from prolonged contact cannot be excluded, it has evidently not given rise to industrial poisoning.

Introduction of an amino group into the phenol molecule yields *amino-phenols* which are intermediates in various chemical industries. The three isomers, para-, ortho- and meta-aminophenol are considerably less toxic than aniline, and the phenol action is also considerably attenuated. Whereas the para compound very readily forms methemoglobin, this is less marked with the ortho and, especially, with the meta compound. The toxic action is somewhat reduced by replacing one hydrogen atom of the amino group by an acetyl radical (acetyl-aminophenol) or by alkyl groups, as in p-methyl-aminophenol and o-methyl-aminophenol which are used as developers in photography and which may give rise to dermatitis. Whether or not this is a direct action or is due to quinone formation does not appear to be settled. The methemoglobinemic action is abolished by substitution of the second hydrogen atom by an ethyl radical (ethyl-acetyl-p-aminophenol).

The toxicity of aminophenol is also reduced by closure of the hydroxy group by etherification, the ethyl ether, phenetidine, being used as analgetic and antipyretic. Of the corresponding acetyl derivative, phenacetine, which has less

marked side actions than acetanilide, a number of homologues have been prepared and studied. Of these the methyl ether is more and the propyl, butyl and amyl ethers are increasingly less toxic so that it appears that this is a function of the stability of the ether binding. Closure of the hydroxy group of acetyl-p-aminophenol by esterification with an acid, as in diacetyl aminophenol, appears to yield less stable and, hence, more toxic compounds.

Introduction of one or more nitro groups into the phenol molecule yields mono-, di- and tri-nitrophenols which play an important rôle in the explosives industry and which are also encountered as intermediates in the chemical industry and sometimes in other industries.

Of the three mono-nitrophenols the para compound is said to be the most and the ortho derivative the least toxic. They cause stimulation and depression of the central nervous system and are methemoglobin formers.

Of the six isomeric dinitrophenols the 1,2,4-dinitrophenol is most commonly used. The 1,2,4-, 1,2,5- and 1,2,6-dinitrophenols are said to be more toxic than the 1,3,5-, 1,3,4- and 1,3,2-derivatives. These substances increase the oxygen metabolism and raise the body temperature, which effect is, at least to some extent, bound to the presence of one nitro group in para position to the hydroxy group. In addition, they may cause stimulation and, later, depression of the central nervous system, visual disturbances (cataract), injury of liver and kidneys, blood changes (agranulocytosis), and irritation of the skin resulting in dermatitis. Whereas one of the most active hyperthermic agents, 1,2,4-dinitrophenol, produces no methemoglobinemia, the less toxic compounds, 1,3,2- and 1,3,6-dinitrophenol, are methemoglobin formers, so it appears that the hyperthermic and methemoglobinemic actions are linked to different parts of the molecule and are possibly caused by different metabolites. Of the alkyl derivatives of dinitrophenol the 4,6-dinitrocresol and 3,5-dinitrocresol have qualitatively and quantitatively an effect very similar to that of 1,2,4-dinitrophenol, the latter of the two being, however, somewhat less toxic than the former.

Introduction of a third nitro group into the phenol molecule yields trinitrophenol. Of the four isomers, 2,4,6-trinitrophenol (picric acid) is the most important, being extensively used in the explosives and dye-stuffs industries. In contrast to the lower nitration products of phenol, with this compound the irritant action on the skin and mucous membranes is the predominant toxic effect. This may lead to dermatitis, conjunctivitis and bronchitis. With heavy exposure it may lead to nervous and gastro-intestinal disturbances, but it does not produce methemoglobinemia. The decrease of the methemoglobinemic action, with subsequent nitration, and also the decrease of the systemic toxicity, is a phenomenon which will also be observed with other substitutions of inorganic radicals in aromatic hydrocarbons.

Introduction of a second hydroxy group into benzene yields *diphenols*, the ortho compound being known as pyrocatechol, the meta compound as resorcinol, and the para compound as hydroquinone. They are used in photography, in the rubber industry, and in the chemical industries. Systemically they cause convulsions of the spinal type, hydroquinone being the least and pyrocatechol

the most effective in this respect. In addition they cause methemoglobinemia, leucopenia and anemia which were also observed in benzene poisoning. Their contact with the skin, especially hydroquinone, may lead to eczema. Some of these effects are evidently affiliated with their ability to form quinones, affecting in this way other oxidation-reduction potentials in the organism. Etherification of one hydroxy group by alkyl radicals reduces the toxicity of diphenols, the mono-methyl ether of pyrocatechol (guajacol) being more toxic than the corresponding hydroquinone derivative, and it appears that with increasing molecular weight the toxic action of diphenol mono ethers decreases, whereas the local action increases.

Introduction of a third hydroxy group into the benzene molecule yields triphenol, and, according to the relation of these to each other, three isomers may be formed. Two of these, 1,2,3-trihydroxy benzene (pyrogallol) and 1,2,5-trihydroxy benzene (phloroglucinol), are used in various industries. Their depressant and convulsant actions are considerably decreased as compared with those of benzene and phenol. The 1,2,3 compound is said to be twenty times more toxic than the 1,2,5 derivative which is practically inactive. The former may cause irritation and erythema of the skin, and when absorbed may cause methemoglobinemia, hemolysis and nephritis with hematuria.

3. The *aldehydes* of the aromatic series, such as benzaldehyde, protocatechu aldehyde and vanilline, are of limited industrial importance. The latter is said to cause headache, vertigo and somnolence during the day; nervousness and insomnia during the night. They are readily oxidized in the organism, resulting in practically inert carboxylic acids.

4. The *aromatic carboxylic acids*, such as benzoic acid and its derivatives, amino and o-hydroxy benzoic acid (salicylic acid), as well as the dicarboxylic acid, phthalic acid, are used in the chemical industry and are of very limited toxicity. Only the anhydride of the latter, phthalic acid anhydride, is of some toxicological importance. It causes irritation of the eyes and the respiratory tract on account of its dehydrating action. Several esters of phthalic acid, dimethyl phthalate, diethyl phthalate and dibutyl phthalate, have recently gained some industrial importance as plasticizers for nitrocellulose and acetyl cellulose. On account of their high boiling point the danger of toxic effects from the inhalation of their vapors seems to be remote. Exposure to a mist of these materials results in irritation of the mucous membranes of the eyes and the respiratory tract. The use of diethyl phthalate as a denaturing agent for alcohol has given rise to irritation of the skin, characterized by itching, eczema and paresthesias of the fingers. It may also have some effect on the blood and blood-forming organs, and may cause injury to the liver and kidneys. However, the mechanism of this action appears to need further study.

5. Of the halogenated benzene derivatives, only the chlorine compounds, mono-chloro-benzene and di-chloro-benzene, are of industrial importance. These are used as solvents for acetyl cellulose, resins and fats, as cleaning agents for metals and as intermediates in the chemical industries. Mono-chloro-benzene causes moderate irritation of the skin and mucous membranes. It

depresses the central nervous system but is said to be less toxic than benzene. Di-chloro-benzene is also used as an insecticide and fumigant and, technically, usually as a mixture of the two isomers, ortho- and para-dichloro-benzene, the latter being considered more toxic. Their depressant effect on the central nervous system appears to be more pronounced than that of the mono derivative. The effect of these substances on the blood pigment, the blood, and the blood-forming organs has not been studied, and a study of their effect on the hemopoietic system, especially in relation to their fate in the metabolism in comparison with that of benzene, and their hepatotoxic action may be of considerable interest and of practical importance.

Introduction of halogens in an aliphatic side chain, as in that of toluene, yielding benzyl chloride, benzyl bromide and benzyl iodide, results in chemicals of extremely irritant character which led to their use as war gases.

The chlorination product of diphenyl, diphenyl chloride, is used in synthetic waxes. It causes irritation of the skin and injury to the liver, being more toxic in this respect than the chlorinated naphthalenes.

6. Introduction of an amino group into the benzene ring yields aminobenzene (aniline) which is used extensively in the chemical and explosives industries, in dyeing, and in the manufacture of rubber articles. It is a blood and nerve poison, causing methemoglobin formation, primary excitation followed by depression of the central nervous system, and, finally, loss of consciousness. With prolonged exposure, anemia, circulatory irregularities, disturbances of the gastrointestinal tract, injury to the kidneys and nervous disturbances may be observed. The methemoglobin formation is not caused by aniline itself but is presumably due to its metabolite, p-aminophenol, and its oxidation product, quinone-imine.

Introduction of alkyl and aryl radicals into the amino group, as in methyl aniline, dimethyl aniline, diethyl aniline, diphenyl aniline and benzyl aniline, yields compounds which are used as intermediates in the chemical industries. The alkyl derivatives differ only quantitatively from aniline, the fundamental toxicological character of the mother substance being unaffected. With the aryl derivatives, on the other hand, not only is the toxicity reduced but the character is also changed, the methemoglobin formation being missing.

Introduction of formic acid and acetic acid radicals into the amino group of aniline yields formanilide and acetanilide which are used in the chemical and rubber industries, the latter being also used as an analgesic and antipyretic in medicine. Their action is, qualitatively, very similar to that of aniline but much less abrupt, and both are methemoglobin formers.

Substitution of the second hydrogen of the amino group of aniline by a methyl group yields methyl formanilide and methyl acetanilide, both of which are more toxic but less effective as antipyretics. Whereas the methemoglobinemic action of the latter is less marked, as compared with acetanilide, this is not the case with methyl formanilide. This may indicate a greater stability of methyl acetanilide.

Introduction of one chlorine atom into aniline yields monochloroaniline, in which the methemoglobin formation is slightly attenuated and the meta and

ortho compounds are apparently slightly less toxic than the para compound. Introduction of a second and third chlorine atom, as in di- and tri-chloroaniline, decreases only slightly the methemoglobinemic action. This is also the case with mono-, di- and tri-chloroacetanilide.

Introduction of one nitro group into aniline yields o-, m- and p-nitroaniline which are intermediates in the explosives industry. Their toxic action does not differ materially from that of aniline. Further nitration, resulting in tetranitroaniline, evidently reduces the toxicity, as is also observed with tri-nitrophenyl-methyl-nitramine, tetryl, but both of these and also dimethyl amino-p-nitrosoaniline cause marked irritation of the skin, leading to papular eruptions and eczema.

Introduction of a second amino group into the benzene ring yields diamino benzene, phenylene diamine. Of the three isomeric compounds, para-phenylene diamine is used as intermediate in the dye industry and forms the main constituent of "Ursol" dyes which are used for dyeing hair and fur. Its methemoglobinemic action is less marked than with aniline, but it causes edema, especially around the neck, irritation of the skin, asthma, and gastro-intestinal disturbances. Large doses may cause convulsions of the spinal type. The methemoglobinemic, edemagenic and irritant actions on the skin have been credited to its oxidation product, quinone diimine. This, in opposition to quinoneimine, the final metabolite of aniline which represents a redox system, is readily oxidized further, and this may explain the less marked methemoglobinemic action. The meta compound is less toxic than p-phenylene diamine and the ortho compound is still less toxic, which may indicate that they have a different fate in the organism. Whereas substitution of two hydrogen atoms of one amino group, resulting in dimethyl-p-phenylene diamine, increases the toxicity considerably, the diethyl derivative is said to be about as toxic as the mother substance, but both materials are more readily absorbed through the skin.

Introduction of one amino group into toluene yields amino toluene or toluidine which is used as an intermediate in the dyestuff industry. It exists as ortho-, meta- and para-toluidine. The toxic action of the ortho compound resembles closely that of aniline, the acetyl derivative is also a methemoglobin former, but the antipyretic and analgetic actions are less marked than those of acetanilide. Whereas meta-toluidine is more toxic than ortho-toluidine, m-acet-toluidine does not form methemoglobin, and the same holds true for p-acet-toluidine although p-toluidine itself is the most toxic of the three isomers. The reason for this difference in their behavior may be their different fate in the organism, the ortho compound being largely oxidized to a phenol and the para compound to p-amino-benzoic acid.

The three monochlor-toluidines, 5-chloro-2-toluidine, 4-chloro-2-toluidine and 6-chloro-2-toluidine, are intermediates in the dyestuff industry. Toxicologically they closely resemble toluidine and aniline. With the 5,2 compound, irritation, congestion and hemorrhages of the bladder were observed.

Of the six isomeric diamino toluenes, only 2,4-diamino toluene has been studied extensively. Its irritant action on the skin is similar to that produced

by p-phenylene diamine. It destroys the blood cells and causes severe injury to the liver. The mechanism of both actions has aroused much interest and it appears to be established that the toluidene diamine icterus is not exclusively of hemato-genic origin.

Amino xylene, which is used as an intermediate in the dyestuff industry, closely resembles aniline and toluidine in its action. Introduction of two amino groups into diphenyl yields diamino diphenyl. The 4,4' compound, benzidine, is mainly important as an intermediate in the dyestuff industry. Its action resembles that of other aromatic amines, but it is less liable than most of these to form methemoglobin. However, it may cause anemia and liver and renal injury and it has been credited with the formation of bladder tumors although the latter has not been demonstrated experimentally.

7. Introduction of one nitro group into the benzene ring yields nitrobenzene which is an intermediate in the manufacture of aniline, aniline dyestuffs and explosives. It is also occasionally used in the manufacture of shoe dyes and as a flavoring agent and cheap perfume. Acute poisoning from its ingestion or from inhalation of high concentrations may cause loss of consciousness and paralysis. Continued exposure to lower concentrations may result in blood changes, cyanosis, and disturbances of the nervous functions and of the gastrointestinal tract with its sequelae. The methemoglobin formation sets in only after a latent period of several hours, presumably because it takes some time to form the metabolites, nitrosophenol and quinoneimine, which are responsible for this effect. The nitrobenzene anemia appears to be primarily due to a destruction of the red blood cells, whereas the effect on the white blood cells is much less conspicuous than with benzene.

Introduction of one chlorine atom into nitrobenzene yields mono-chloro-nitrobenzene which is used in the explosives industry. Qualitatively it resembles nitrobenzene in its toxic action but quantitatively it is considered more dangerous.

Introduction of a second nitro group into nitrobenzene yields dinitrobenzene. Of the three isomeric compounds the meta derivative is the most important, being an important intermediate in the dyestuff industry and an explosive. Exposure to this material may cause anemia, cyanosis, nervous disturbances and injury to the liver and the kidneys. Its methemoglobinemic action is less marked than that of mono-nitrobenzene and, like the latter, it affects mainly the red blood cells and to a lesser extent the white blood cells. Its chlorination product, mono-chloro-dinitrobenzene, is also used as an explosive and its systemic action is very similar to that of dinitrobenzene. It causes, however, very marked irritation and is considered more dangerous than the latter. This irritant effect is very severe with the higher chlorination product, dichloro-dinitrobenzene, with which contact with the skin may result in erythema, edema and necrosis, whereas little is known in regard to its systemic effect.

Introduction of a third nitro group into dinitrobenzene yields trinitrobenzene which has a toxicological action similar to that of dinitrobenzene. It is said to form methemoglobin but it does not appear to be established whether or not

there are quantitative differences between the di and tri nitration product in this respect.

Introduction of a nitro group into the toluene molecule yields mono-nitro-toluene which is an intermediate in the dyestuff and explosives industries. Toxicologically its action is similar to that of nitrobenzene, but whereas the ortho compound is said to be about as toxic as the latter, the para compound is considerably less toxic.

Of the six isomers of dinitrotoluene the 2,4-dinitro compound is the most important, being used in the explosives industry. The pure compound is said to be less toxic than dinitrobenzene, usually causing only general complaints about headache, fatigue, dizziness, moderate anemia and cyanosis.

Of the five isomers of the triple nitration product of toluene, 2,4,6-trinitro-toluene is the most important, being widely used as a high explosive. The literature on the toxicity of the technical product is highly controversial and it appears that the impure material may be considerably more toxic as compared with the relatively low toxicity of the pure compound. Persons handling trinitro-toluene frequently show yellow discoloration of skin and hair, and they suffer from irritation of the mucous membranes and the skin which may develop into various types of dermatitis. Exposure to trinitrotoluene may result in nervous disturbances of general nature or in neuritides, irregularities of the circulation, disturbances of the gastro-intestinal tract, atrophy of the liver, injury to the kidneys, anemia and cyanosis. The two latter may be the only and the most conspicuous phenomena and may be the cause of the icterus. The anemia is mainly characterized by a destruction of the red blood cells, whereas the effect on the white blood cells may be characterized by leucopenia and leucocytosis but is most commonly characterized by a relative lymphocytosis. The cyanosis appears to be due partly to the formation of methemoglobin and partly to the formation of pigments formed from trinitrotoluene in the organism.

The nitration products of xylene appear to be less toxic than those of toluene and benzene, mono-nitroxylene being considerably less toxic than nitrobenzene, dinitroxylene less than dinitrobenzene and dinitrotoluene, and trinitro-, and especially tetranitroxylene being only very little toxic. It appears, therefore, that the presence of one methyl group reduces the toxicity of the aromatic nitro compounds, and the presence of two methyl groups reduces it further.

8. Introduction of a hydrazine radical into the benzene molecule yields phenylhydrazine which is used in laboratories and in the chemical industries. This material should be handled very carefully as contact with the skin causes erythema, itching, pustulous and papulous eruptions and edema. Continued exposure may lead to sensitization, resulting in allergy characterized by gastro-intestinal disturbances and attacks of asthma. Larger doses may cause irritation of the kidneys, anemia and cyanosis. The destruction of red blood cells is said to be more marked than was observed with other aromatic amino compounds, as, for instance, diaminotoluene, and for this reason phenylhydrazine has been used therapeutically in the treatment of polycythemia. The cyanosis is partly due to the formation of a pigment from phenylhydrazine and partly caused by formation of a methemoglobinlike substance which evidently is not identical

with ordinary methemoglobin. The higher homologue, toluyldiazine, is said to be less destructive to red blood cells, and the meta derivative, but not the ortho and para compounds, causes facial edema, as observed with paraphenylenediamine.

C. NAPHTHALENE AND ITS DERIVATIVES. Naphthalene, a hydrocarbon consisting of a double benzene ring, is handled in various chemical industries and especially in the dyestuff industries, and it is also used as an insecticide. Being solid and not very volatile, it has comparatively little toxic effect from the point of view of industrial exposure. Inhalation of low concentrations may cause headache, somnolence, and loss of appetite. With more severe exposure these effects become more marked and there may be, in addition, some injurious effects on the kidneys, resulting in albuminuria and hematuria. Its contact with the skin causes irritation, resulting in itching, erythema and eczema. It is also irritant to the mucous membranes of the respiratory tract and the eyes. On the latter it may cause punctate turbidities of the cornea, cataract and, occasionally, optic neuritis. Its destructive effect on the blood is not marked and certainly not comparable to that of benzene.

Its hydrogenation products, tetrahydro- and decahydronaphthalene, tetraline and decalene, are liquids of high boiling point which are used as solvents for fats and waxes. They are only slightly toxic and their toxic character is similar to that of naphthalene.

1. Introduction of one *hydroxy group* into the naphthalene molecule yields two isomeric naphthols which have qualitatively the same toxic effects. They are important intermediates in the dyestuff industry. They are readily absorbed through the skin and cause irritation of the skin and the mucous membranes of the respiratory tract and the eyes, causing conjunctivitis, temporary turbidity of the cornea and, after absorption of larger amounts, cataract and injury of the retina. Absorption of sufficient quantities may lead to irritation of the kidneys, resulting in the excretion of albumen, blood and hematin.

2. Chlorination of naphthalene leads to tri-, penta- and hexachloronaphthalene which are constituents of synthetic waxes. Their toxicity increases with the amount of chlorine introduced and they cause irritation of the skin, characterized by itching, pruritus, erythema, vesicular-edematous dermatitis, and chloracne. Whereas trichloronaphthalene is comparatively innocuous in low concentrations, penta- and hexachloronaphthalene are much more toxic and may cause severe injury to the liver.

3. Introduction of an *amino group* into the naphthalene molecule yields alpha and beta naphthylamine which are important intermediates in the dyestuff industry. The alpha compound is said to be less toxic than the beta compound, especially in regard to the production of bladder tumors which are presumably caused by some metabolite of the latter and which are frequently preceded by strangury and hemoglobinuria. Of the hydrogenation products of naphthylamine the *alpha*-tetrahydronaphthylamine is of interest because it increases the body temperature and the oxygen metabolism. In sufficiently large doses it may cause nausea, vomiting, headache and impaired respiration.

The diamino compound of naphthalene is an intermediate in the dyestuff

industry. It is said to be only slightly toxic and, especially, to be free of the edemagenic action observed with *p*-phenylene diamine.

4. The *nitration* products of naphthalene, mono- and dinitronaphthalene, both of which are intermediates in the dyestuff industry, are comparatively only slightly toxic, only the absorption of large amounts causing irritation of the kidney. Their fumes are irritant to mucous membranes and may occasionally lead to injury of the cornea.

The double nitration product of naphthol, Martius yellow, has caused no severe poisoning in humans. In animals, comparatively large doses cause irritation of the gastro-intestinal tract and the kidneys, and fever, but no marked effect on the blood and the blood-forming organs.

D. OTHER ORGANIC COMPOUNDS. Of the heterocyclic organic compounds, pyridine is used as a solvent in chemical industries and as a denaturing agent for alcohol. The latter use is said to have caused irritation of the skin and eczema. Its vapors are irritant to the mucous membranes, especially those of the eyes, and high concentrations may cause headache, fatigue, nausea, loss of appetite, diarrhea, general nervous disturbances, neuritides and loss of consciousness.

Thiophene, the corresponding sulfur compound, is one of the contaminants of crude benzene. Its action is similar to but not quite so marked as that of benzene, especially in regard to its effect on the blood and the blood-forming organs.

None of the polycyclic heterocyclic compounds is of great industrial toxicological importance. Quinoline, which is a starting material in the dyestuff industry, causes in high concentrations some depression of the central nervous system, and acridine, the mother substance of the various acridine derivatives, causes irritation of the skin and the mucous membranes, causing sneezing, coughing and conjunctivitis.

A review such as that presented in the foregoing has necessarily to be restricted to an outline of the toxicological action. In many instances only those substances which are either very toxic or important as therapeutic agents have been studied. The understanding of the mechanism of action of many of these chemicals would be much improved if the outstanding toxicological characteristics could be followed in chemically related compounds, as has been done in a few instances, and if their fate in the metabolism could be studied more closely, especially with regard to their chemical constitution. For the evaluation of their potential dangers with industrial exposure, continued exposure experiments with doses or concentrations similar to those which might be encountered in industry should be studied more extensively, special emphasis being placed on possible nervous and blood changes, and pathological changes of the metabolism and vital organs.

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CHEMOTHERAPY OF AVIAN MALARIA

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The investigation of drugs for their antimalarial action is to a large extent limited to avian infections. Experimental animals for investigations of the chemotherapy of malaria are restricted to various species of birds and monkeys. For a number of reasons, large scale use of the latter is impractical. The present review is confined to the chemotherapeutic aspects of avian malaria: no attempt has been made to include other studies on avian malaria which have contributed to our knowledge of the disease.

METHODS. The canary infected with *Plasmodium relictum* has been the experimental animal most commonly used. However, several other species of *Plasmodium* have been utilized in the canary. Russian investigators have used *P. relictum* infections in small birds other than the canary. Recently, chemotherapeutic studies have been made on infections of *P. gallinaceum* in the chicken, *P. lophurae* in the chicken and duck, and *P. relictum* in the pigeon. *Haemoproteus orizivora* (not a malarial parasite), a naturally occurring infection in the Java sparrow, has been used: only gametocytes occur in the blood, and schizonts are restricted to the endothelium of the internal organs.

Infection has been produced by inoculation of trophozoites (intraperitoneally, intramuscularly or intravenously) or of sporozoites. The latter method has been used only to a limited extent in chemotherapeutic investigations and has been confined to infections of *P. relictum* and *P. cathemerium* in the canary and of *P. gallinaceum* in the chicken. Little attention appears to have been given to the quantitative dosage of parasites in experimental infections. The infection in the young duck produced by the intravenous injection of blood heavily infected with *P. lophurae* offers many advantages over the other infections which have been used for chemotherapeutic studies (Hegner, West, Ray and Dobler, 1941; Coggeshall, 1941; Walker and van Dyke, 1941; Marshall, Litchfield and White, 1942).

Administration of drugs has been by injection (subcutaneously, intramuscularly or intraperitoneally), by stomach tube, and by incorporation in the food. Schedules of doses have varied: the commonest used is that of Roehl (1926), i.e., one dose daily for six days. Very little information is available as to the optimal method of therapy in avian malarial infections. From analogy to what has been established in experimental bacterial chemotherapy, there is reason to believe that frequently repeated doses may be much more effective than a single daily dose although the total amount of drug is the same in both instances. The drug-diet method (Bieter et al., 1940; Litchfield, White and Marshall, 1939), by which a more or less constant blood concentration of drug is maintained, appears to be useful in the treatment of avian malarial infections (Coggeshall, 1941; Walker and van Dyke, 1941; Marshall, Litchfield and White, 1942).

The responses which have been used as criteria of antimalarial activity of

drugs are: 1, reduction of the blood parasite concentration-time curve; 2, significant delay in the incubation period (time of appearance of parasites in the blood), and 3, decrease in the relapse rate. In infections, where mortality of untreated birds is high, survival time or percentage survival may have advantages over the responses mentioned above.

The quantitative methods for comparing the activity of drugs so far published are not entirely satisfactory. Roehl (1926) estimated the activity of a drug in terms of the minimum dose which caused a delay in the appearance of the parasites in the blood. He then determined the maximum tolerated dose of drug for the canary, and compared drugs on the basis of the chemotherapeutic index. Fourneau and co-workers (1931), using the *Haemoproteus* infection of the Java sparrow, determined the fraction of the maximum tolerated dose which just produced a definite decrease in the parasitemia. This was a measurement of effect on the gametocytes of a parasite which is not a member of the genus *Plasmodium*. Buttle, Henry and Trevan (1934) used groups of 6 canaries; one group was left untreated; one group received one dose per day for 6 days of quinine, and other groups received the same doses of different drugs. The mean time before the parasites appeared in the blood was determined for each group. These authors emphasized the importance of using in each experiment a group of birds treated with quinine to serve as a standard of comparison, and attempted to calculate the error of the comparisons.

There are several objections to the above methods. It is questionable whether the chemotherapeutic index of a drug determined for the bird is of any value in assessing the drug for clinical use. Thus, although plasmochin has a much higher chemotherapeutic index than quinine in the bird, it is well known that with plasmochin the margin of safety in the human is too small to allow its extensive clinical use. Differences in absorption, excretion and toxicity of different drugs for the bird as compared to the mammal are almost certain to invalidate the transference of a chemotherapeutic index from one class to the other. Buttle and co-workers (1934), whose quantitative method appears to be the best of the three mentioned above, used the bird solely for assessing the comparative antimalarial value of the drugs, and performed toxicity determinations of the drugs on mice. This procedure of using the bird for assaying the therapeutic effect, and mammals for the toxic effect, appears to be the most satisfactory available at present.

Another very probable source of error in these quantitative methods is the difficulty of obtaining an accurate comparison of drugs on a single daily dosage schedule. Differences in the absorption, excretion, and destruction of two drugs may render the comparison misleading even for the bird. An example of such errors in the comparison of the activity of two drugs on the single daily dosage schedule may be given from data obtained in bacterial chemotherapy. Thus, when diaminodiphenylsulfone and sulfanilamide were tested on a streptococcus infection in mice by the single daily dose method, the former drug was found to be 100 times as active as the latter; when tested on the basis of maintained blood concentrations of the two drugs, their activity ratio was only 3 to 1 (Marshall,

Litchfield and White, 1940). On the basis of experiments on sulfonamide therapy in duck malaria, Marshall, Litchfield and White (1942) suggest that the antimalarial activity of drugs can be assessed quantitatively by determining the blood concentration of each drug which gives the same response. They suggest as the responses to be used parasitized cell counts, survival time or percentage survival as these three responses are highly correlated with each other. Even where it is impossible to determine the blood concentrations of drugs, a comparison of activity, based on a dosage schedule, by which more or less constant blood concentration is maintained, would appear to be less in error than one based on single daily doses.

Just how much the single daily dosage schedule may invalidate conclusions as to the qualitative antimalarial activity of drugs is not known. There are several reports stating that drugs, which showed activity in simian and/or human malaria, were completely inactive in avian malaria (Christophers and Fulton, 1938; Fulton, 1940). Possibly if these drugs were retested with a more efficient dosage schedule some activity would be found. At least, several sulfonamide drugs, which have been reported as inactive in avian malaria when tested by the single daily dose schedule (Coggeshall, 1938; Buttle, 1939; Manwell, Counts and Coulston, 1941; Hegner, West and Dobler, 1941), have been found active when examined by the drug-diet method (Coggeshall, 1941; Walker and van Dyke, 1941; Marshall, Litchfield and White, 1942).

Therapeutic effect of quinine and other drugs. A number of investigators have studied the therapeutic effect of a variety of drugs in avian malarial infections produced by injection of infected blood (trophozoite infection). Kopanaris (1911) found quinine had a curative action but no protective action (when mixed with the inoculum) on *P. praecox* infections in the canary, and that arsphenamine and atoxyl were without effect. Marks (1914) saw no effect on canary malaria from the injection of methylene blue, but a marked effect when the drug was given in the bird's food. The Sergeant brothers (1921) carried out the first extensive study of the action of quinine in canary malaria. They differentiated between "curative" and "preventive" treatment, worked out non-toxic doses of quinine, and showed that the drug did not eradicate the infection. They also found that the same amount of quinine given as three doses a day was less toxic and more effective than when given as a single dose. For prevention, they found it useless to start treatment before infection, but necessary for the best result to start on the day of infection. Also, when given every day the drug was more effective than when given every other day or once a week. When quinine was given every day or every other day for 1 to 9 months, the parasites in 1 bird became quinine resistant; in 2, they remained virulent; in 2, they were destroyed; and in 3, they were attenuated in virulence.

Further studies of the effect of quinine or other alkaloids of cinchona bark either in delaying the appearance of the infection or of decreasing an already established infection of canaries have been reported by the Sergeants and Catanei (1923, 1924), Sergeant and Catanei (1925), Ainley and King (1938), Boyd (1925), Giemsa, Weise and Tropp (1926), Morgenroth, Abraham and Schnitzer (1926),

Kikutli and Tropp (1927), Katahira (1929), Fourneau and co-workers (1930), Goodson, Henry and Macfie (1930), Giemsa and Oesterlin (1933), Buttle, Henry and Trevan (1934), Missiroli (1937), Cohen and King (1938), and Prelog, Stern, Seiwert and Heimhaeh-Juhász (1940). Attempts have been made by many of the above workers to assess the comparative therapeutic efficiency of the alkaloids of cinchona bark. Giemsa, Weise and Tropp (1926) compared the action of various quinine derivatives on canary malaria with that on human malaria. Table 1 gives their results.

The naturally occurring *Haemoproteus* infections in the Java sparrow and pigeon (where gametocytes only are present in the blood) were used by the Sergents (1907) and by Collier and Krause (1929) for testing antimalarial drugs. This method has been developed extensively by Fourneau and co-workers (1931). They found quinine to have no effect; stovarsol and methylene blue were quite active. A number of 8-amino quinoline derivatives were tested by

TABLE 1

| DRUG | CANARY MALARIA DELAY IN APPEARANCE OF PARASITES OVER CONTROLS | HUMAN MALARIA ACTIVITY |
|--------------------------|--|---------------------------|
| | days | |
| Cuprein..... | 0-4 | 0 or + |
| Quinine..... | 12-13 | ++ |
| Quinidine..... | 12 | ++ |
| Chinchonin..... | 0-5 | + |
| Chinpropyrlin..... | 1-4 | + or ++ |
| Hydroquinine..... | 11 | ++ or +++ |
| Optochin..... | 6-11 | + or ++ |
| Chitenin..... | 0 | 0 |
| Chitenin-ethylester..... | 5-12 | + or ++ |
| Chinicin..... | 0 | 0 |

Fourneau, Tréfouels, Bovet and Benoit (1933); quinine and atabrine were found inactive by Bovet and Demnehe (1933). Bovet, Benoit and Altman (1934) found the chemotherapeutic index of 8-diethylamino-undecylamino-6-methoxy-quinoline to be 3 on *Haemoproteus*, but 100 on *Plasmodium* in the canary. The action of drugs on *Haemoproteus* infections has also been studied by Moehkovski (1935), Albriecht and Nieuwenhuys (1937), and Kikuth (1938). Durand and Villain (1939) found sulfanilamide to have no effect on *Haemoproteus* infections in pigeons.

Drugs, other than quinine and its derivatives, have been investigated as to their therapeutic effect in canary malaria. In 1926 Roehl introduced plasmoehin (Schulemann, 1932). He found it to be a much more potent drug than quinine, and to have a much higher chemotherapeutic index for *P. relictum* infections in the canary. Hegner and Manwell (1927) confirmed the efficacy of plasmoehin in *P. cathemerium* infections of the canary. Other studies on quinoline compounds related to plasmoehin are reported by Collier and Warstadt

(1931), Fournneau et al. (1930), Sergeant and co-workers (1931), John and Glowasky (1933), Magidson and Strukow (1933), Kritschewski and Sternberg (1933), Tate and Vincent (1933), Sternberg (1934) and Magidson, Madazema and Rubzow (1935). Atabrine, introduced by Kikuth (Kikuth, 1932; Kikuth and Schönhöfer, 1935), acts strongly on canary malaria, but not upon the *Haemoproteus* infection of the finch. Recently, Kikuth (1938) announced a new antimalarial of the quinoline type, "Certuna" (α -dialkylamino-oxyquinolyl-butane).

Stovarsol, which is stated to be effective in benign tertian malaria of man, appears to have no action on *P. relictum* infections in the canary (Sergeants and Catanei, 1925; Fournneau et al., 1930). Methylene blue, which has some effect on the quartan human infection, possesses only a slight action on *P. relictum* in the canary, but has a marked action on *Haemoproteus* in the Java sparrow (Fournneau et al., 1930; Bovet and Demanche, 1933; Albricht and Nieuwenhuys, 1937). Mercurochrome, reported to be of some value in benign tertian malaria, appeared to show some effect in *P. cathemerium* infections of the canary (Swezey, 1935). The few amidines which have been tested are reported to be inactive in canary malaria (Easson and Pyman, 1931; Christophers and Fulton, 1938; Fulton, 1940).

Although sulfanilamide was first reported to be of value in the treatment of human malaria, these promising reports have not been confirmed. In both acute and chronic infection with *P. knowlesi* in the monkey, Coggeshall (1938, 1940) has shown that sulfanilamide effects a complete and permanent cure. In *P. relictum*, *P. cathemerium*, and *P. nucleophilum* infections of canaries, neither sulfanilamide nor sulfapyridine were found to have any effect (Coggeshall, 1938; Buttle, 1939; Manwell, Counts and Coulston, 1941): in *P. relictum* infections, a number of sulfanilamide derivatives with side chains resembling that of plasmochin were reported as inactive (Walker, 1940). In contrast to these negative results are the observations that neoprontosil caused complete disappearance of parasites in *P. praecox* (?) infection in Java sparrows (Africa, Dy and Soriana, 1939) and that sulfapyridine had a favorable effect on *P. circumflexum* infections of the canary (Manwell, Counts and Coulston, 1941).

Very few chemotherapeutic studies on infections in avian hosts other than the canary have been reported. Russian workers (Kritschewski and Rubinstein, 1932; Kritschewski and Sternberg, 1933; Mochkovski, 1935) report the use of *P. relictum* in *Acanthis linaria*, *Spinus spinus*, and *Fringilla linaria*. Brumpt, Bovet and Brumpt (1937) tested the effect of drugs on *P. gallinaceum* in the chicken. Coatney and Young (1939) studied the action of colchicine on *P. relictum* infection of pigeons. The activity of quinine and hydroxyethyl-apocupreine has been reported in ducks infected with *P. lophurae* and in pigeons with *P. relictum* (Hegner, West, Ray and Dobler, 1941; Hegner, West and Dobler, 1941). In *P. lophurae* infections of chickens and ducks several sulfonamides were reported to be entirely without influence on the infection (Coggeshall, 1938; Hegner, West and Dobler, 1941). However, with the use of the drug-diet method of therapy, sulfonamides have been found to be active in this

duck infection (Coggeshall, 1941; Walker and van Dyke, 1941; Marshall, Litchfield and White, 1942).

Action of drugs on different stages of parasite. It has been assumed that there are four stages in the life-cycle of the malarial parasites where a drug may act: 1, on sporozoites; 2, on trophozoites; 3, on gametocytes, and 4, on hypothetical forms responsible for relapse (Schulemann, 1932). All of the chemotherapeutic investigations discussed above have dealt with trophozoite infections (injection of blood of an infected bird into a clean bird) or naturally occurring gametocyte infections of the blood stream. Very few chemotherapeutic studies have utilized sporozoite infections or mosquito transmission of the disease. Manwell (1932, 1934) stated that in trophozoite infections of the canary with various species of *Plasmodium*, treatment with quinine and plasmochin was much more effective when started at the time of infection than if delayed until parasites were present in the blood. Missiroli (1937) also found that quinine was more effective if given during the incubation period than if given when parasites were present in the blood. However, Brumpt, Bovet and Brumpt (1937) stated that with mosquito infections of *P. gallinaceum* in the chicken, quinine, plasmochin and atabrine were curative in the sense that when administered after the parasites had appeared in the blood, they markedly decreased the infection. However, if treatment with these drugs was started at the time of the infection, the incubation period was not prolonged.

Is any drug a prophylactic in the sense of preventing the infection? Russell (1931) stated that plasmochin was an effective prophylactic against *P. calhemerium* trophozoite infection in the canary: Hogner and Manwell (1927) did not find this to be true. On the other hand, Russell (1932) found that plasmochin was not a prophylactic for infections of *P. calhemerium* produced by mosquito bites or by injection of sporozoites. Also, Russell (1934) stated that atabrine did not have a prophylactic action in *P. relictum* infections caused by injection of sporozoites. On the other hand, the Sergeants (1922) working with *P. relictum* in the canary found that quinine given immediately after an infection produced by the injection of sporozoites was more effective than in an infection produced by trophozoites. However, if mosquitoes were used, the drug was less effective. Tate and Vincent (1933, 1934) in two very carefully controlled investigations showed that plasmochin and atabrine were very effective in prolonging the incubation period or preventing infection in canaries with *P. relictum* when blood inoculation was used but had no effect on the incubation period when infection was caused by bite of infected mosquitoes. They stated, however, that with the mosquito infection the disease was milder after the administration of atabrine than in the controls. Missiroli (1937) found a definite influence of quinine on a *P. relictum* infection produced by injection of sporozoites in canaries: his data indicated no difference between the effect of quinine in the sporozoite infection and one produced by trophozoites. Godoy and Lacorte (1928) found that plasmochin caused a disappearance of gametocytes from the blood of pigeons infected with *Haemoproteus*, and also acted on sporozoites. They believed that the action of a drug on gametocytes was correlated with its

action on sporozoites. Wampler (1930) found that plasmochin caused first a disintegration of trophozoites of *P. cathemerium* in the canary and later an effect on the gametocytes.

Very few reports of the effects of drugs on the exoerythrocytic stages of avian malarial parasites (Porter and Huff, 1940) have appeared. Neither quinine nor atabrine appeared to have any appreciable effect on the exoerythrocytic forms of *P. gallinaceum* (James and Tate, 1937; Raffaele, 1938): atabrine was without influence upon those of *P. relictum* (Rodhain, 1939). Kikuth and Mudrow (1939) studied the therapeutic effect of quinine, atabrine, "Certuna," and plasmochin on the exoerythrocytic forms of the virulent strain of *P. cathemerium*. They found that plasmochin was the only one of the four drugs which appeared to effect these stages directly.

Evidently, much more investigation is needed concerning the action of drugs on different stages of the parasite to establish the facts and clear up apparent discrepancies. The question of specificity of drugs for sporozoites, gametocytes and trophozoites is a fundamental problem. Are different kinds of drugs needed to produce cure of the acute infection, to prevent relapse, and to act as causal prophylactics?

Species and strain variation in susceptibility to drugs. It is stated that the species of parasites causing human malaria react differently to drugs. Thus, organic arsenic compounds are effective on *P. vivax*, but have no influence on *P. falciparum*; methylene blue acts strongly on *P. malariae*, very little on *P. vivax* and not at all on *P. falciparum*; quinine is most effective against *P. vivax*, but relapses from quinine are greatest with *P. vivax* and least with *P. falciparum* (Schulemann, 1932). It might be expected that differences would exist in the susceptibility to drugs of different species and even strains of avian malarial parasites. The differences in the response of various species of avian *Plasmodium* to drugs has been especially studied by Manwell (1930, 1932, 1934). He found that different strains of *P. relictum* reacted to treatment essentially the same whether quinine or plasmochin was used but that *P. relictum*, *P. rouxi*, *P. cathemerium*, *P. circumflexum*, and *P. elongatum* in the canary showed marked differences in susceptibility. Plasmochin was uniformly superior to quinine, but the degree of superiority depended on the species used. Kikuth (1931) also found that *P. relictum*, *P. cathemerium*, *P. elongatum*, and *P. circumflexum* varied in susceptibility to quinine and plasmochin. Manwell (1933) stated that atabrine does not prevent or cure infections with *P. cathemerium* or *P. circumflexum* but it was a perfect specific against infections with *P. rouxi*. Manwell and Haring (1938) also compared the effect of atabrine and plasmochin in *P. vaughani* infections in the canary. Rauwolfine is stated (Manwell, 1933-34) to have no action on *P. elongatum*, *P. relictum*, or *P. rouxi* infections in the canary, but to have a slight effect on those due to *P. cathemerium*.

Brumpt and Bovet (1936) compared the action of various drugs on the *Haemoproteus* infection of the finch as well as on an induced infection with *P. paddae* in the same animal. They correlated these results with data on *P. relictum* infections of canaries. Table 2 shows the results. Recently, Manwell, Counts

and Coulston (1941) found that sulfapyridine was without influence on infections caused by *P. relictum* and *P. nucleophilum*, but influenced that caused by *P. circumflexum* in the canary. Very little information has been published on differences in susceptibility to drugs of strains within a species. Lourie (1934) observed a considerable difference between two strains of *P. relictum* in canaries in their response to identical dosage schedules of quinine.

It is important to have more data in regard to species and strain susceptibility to drugs. What avian malarial species resembles most closely in its susceptibility to drugs the species producing infections in man? The answer to this question involves a study of the species and strain susceptibility to drugs of the parasites available for experimental infections and a comparison of the data obtained with what is known or can be found as to the action of the same drugs in the three types of human infections.

Can drugs completely cure avian infections? As stated above, the Sergents in their first fundamental work on the chemotherapy of avian malaria found that quinine did not cure consistently a *P. relictum* infection in the canary. Two

TABLE 2

| DRUG | <i>P. FADDAE</i> | <i>HAEMOPROTEUS</i> | <i>P. RELICTUM</i> |
|-----------------|------------------|---------------------|--------------------|
| Quinine..... | + | 0 | + |
| Atabrine..... | + | 0 | + |
| Plasmochin..... | + | + | + |
| 710 F*..... | + | + | + |
| 852 F†..... | 0 | + | + |

+ = Definite therapeutic effect; 0 = No therapeutic effect.

* 8-Diethylaminopropylamino-6-methoxyquinoline.

† 8-Diethylaminoundecylamino-6-methoxyquinoline.

criteria of "cure" were introduced by these workers: namely, 1, no infection from injection of blood from the "cured" bird into a clean bird, and 2, reinfection of the "cured" bird with the same strain and species of parasite. The data of several investigators show that occasional canaries infected with *P. relictum* were sterilized by quinine (when the above criteria of cure are adopted). Manwell found that *P. cathemerium* infections were not sterilized, and Lourie (1934) stated that daily injections of quinine in these infections for nine months failed to sterilize the birds. Hegner and Manwell (1927) found that plasmochin failed to effect a "cure" in *P. cathemerium* infections. Manwell in a series of papers stated that plasmochin sterilized 100 per cent of canaries infected with *P. rouxi* and *P. elongatum*, about 25 per cent of those with *P. relictum* and *P. circumflexum* and none of those infected with *P. cathemerium*; quinine was much less effective as a sterilizing agent in all species.

Drug-fastness. No satisfactory evidence for drug-fastness has been presented. The Sergents (1921) described a quinine-fast strain in 1 canary with *P. relictum* and Kritschewski and Halpern (1933) stated that quinine-fast strains of *P. relictum* when passed through the mosquito sometimes lost and sometimes re-

tained their fastness. However, no good proof of fastness was given and Lourie (1935) in a careful investigation of the matter found no evidence of drug-fastness to quinine of *P. cathemerium*.

In vitro studies. Since a method of cultivating avian malarial parasites *in vitro* is not available, *in vitro* studies have not been extensive or satisfactory. The effect of mixing infected blood with quinine or other drugs and incubating for various lengths of time before injecting into clean birds is reported by several workers (Kopenharis, 1911; Hegner, Shaw and Manwell, 1928; Borchardt, 1930; Lourie, 1934). Borchardt incubated canary blood infected with *P. relictum* with a drug for 5 hours at 37°. Minimum concentrations which prevented infection when incubated blood was injected into canaries were: quinine, 50; hydroquinine, 400; and plasmochin, 20 mgm. per cent. Lourie (1934) found that *P. cathemerium* would resist 1 hour's incubation at 39° with 200 mgm. per cent of quinine. Anschütz (1910) found quinine and methylene blue produced on avian malarial organisms *in vitro* only slight microscopic changes. The recent study of Trager (1941) on the conditions affecting the survival *in vitro* of *P. lophurae* might be utilized with advantage in further studies of the *in vitro* action of antimalarials.

Kikuth (1938) has recently proposed a test for antimalarial activity in which the concentration of a drug which *in vitro* prevents exflagellation of male gametocytes is determined. He found that the results do not agree with those obtained with Roehl's method in canaries, but do agree with those found in the treatment of *Haemoproteus* infections in the finch.

Although the effect of drugs on the oxygen consumption of monkey malarial parasites *in vitro* has been studied (Fulton and Christophers, 1938) similar studies with avian malarial parasites are just beginning (Coggeshall and Maier, 1941). These preliminary studies indicated that inhibition of oxygen consumption *in vitro* alone cannot be depended upon to furnish an index of chemotherapeutic efficiency.

Mechanism of action of drugs. The difficulty of cultivating plasmodia *in vitro* is a definite handicap in investigations in this field. Quinine, and to a much lesser extent, plasmochin and atabrine, are the drugs which have been used in attempts to elucidate the mechanism of action in avian malaria. The simplest theory of mechanism is a direct plasmodicidal effect. Much higher concentrations of quinine than are ever obtained in the blood of treated animals appear to be necessary to kill the parasite *in vitro*. Roskin and Romanowa (1931, 1934) stated that ultraviolet rayed quinine was more effective than unrayed quinine, and that the organic arsenicals were no more effective when used with ultraviolet radiation than when used alone. These data might be used to support the idea of quinine being changed by the host to an active product, but there is no further evidence for this theory of action. The Sergeants (1922) found that an extract of the spleen of a mammal treated with quinine had no therapeutic effect in avian malaria. Kritschewski and Demidowa (1934-35) stated that blockade of the reticulo-endothelial system with trypan blue decreased the effectiveness of quinine, plasmochin and atabrine. Oesterlin (1937) claimed that

dyes like methylene blue depend on the oxidation-reduction potential for action in bird malaria. Fischl and Singer (1934-35) stated that atabrine could be recognized in plasmodia by the fluorescent microscope. It has been claimed that active drugs are only found among those with a high partition coefficient for red blood cells (Hegner, Shaw and Manwell, 1928; Shaw, 1928). Fulton (1937) could not confirm this hypothesis.

Lourie (1934) found that quinine administered to canaries with *P. cathemerium* infections interfered with the normal growth and reproduction of the parasites and upset the characteristic synchronicity of the asexual cycle. These changes did not occur when the powerful influences of the body defenses brought about disappearance of parasites from the blood at the crisis. He, therefore, believed that quinine acted primarily on the parasite and not on body defenses. Boyd and Dunn (1939, 1941) also found that quinine, plasmochin and atabrine, when administered to canaries infected with *P. cathemerium*, inhibited the occurrence of reproduction and brought about a decrease in the size of merozoite group.

Although no conclusion as to the mechanism of action of quinine in avian malaria can be drawn from the scanty data available at present, it appears to the reviewer that Lourie's idea of a primary action of the drug on the parasite and not an action of the drug on body defenses will prove to be correct. This is almost certainly the mechanism of action of the sulfonamide drugs against bacterial infections (Marshall, 1941). It is to be noted that lower concentrations of these drugs are required for effective therapy *in vivo* than those required to kill bacteria *in vitro*. The intimate mechanism of the action of the sulfonamide drugs in duck malaria may be similar to their action in bacterial infections, because the action of these drugs was antagonized by p-aminobenzoic acid in both infections (Marshall, Litchfield and White, 1942).

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ERRATA

The authors of the paper on The Insensible Loss of Water, p. 1, volume 22, no. 1 (January 1942) desire to record the following corrections:

- p. 6:* The column at the right of figure 1, designated "grams," should start at 517 and increase by 345 for each interval. In the legend of this figure the phrase "or grams of water vapor per 24 hours" should be transferred to the end of the last sentence.
- p. 14:* Reference (67) following "Rubner" should become (65).
- p. 16:* Reference (68) should be deleted.



PHYSIOLOGICAL REVIEWS

VOL. 22

JULY, 1942

No. 3

THE VISUAL CENTRES OF THE BRAIN AND THEIR CONNEXIONS

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In recent years a considerable amount of experimental work has been carried out with a view to establishing the finer details of the representation of the retina in the lower and higher visual centres of the brain and to tracing the route by which retinal impulses are conveyed to their destination, and some of the earlier conceptions regarding the diencephalic and mesencephalic terminations of the optic tract have been shown to be erroneous. This work has been necessitated partly by the requirements of experimental psychologists who have made use of visual reactions in their studies of the process of learning and habit formation. The effect of light stimuli on gonadal and other activities has further aroused interest in the anatomical basis of visual reactions.

The structural organization of the retina. Since the classical studies by Cajal, the neuronal structure of the retina in the higher mammals had not received further attention until the histological investigations of Poliak in 1936 (75). Using the Golgi technique, this author made a number of original observations on the conducting units of the retina in monkeys and apes which clearly have an important bearing on the physiology of vision. The most pertinent of these observations are briefly as follows. While the rod elements are all related to the diffuse type of bipolar cell (i.e., several rods are related to one bipolar cell and there is some degree of overlap), the cones are synaptically related to both the diffuse and the individual types of bipolar cell. In the case of the latter, the relationship is mostly monosynaptic, particularly in the fovea and macula, each cone pedicle directly touching the dendritic tuft of a single bipolar cell. In the case of the diffuse varieties, even in the macula, each bipolar cell is related to several cones (and simultaneously to a group of rods). Poliak recognizes four varieties of the diffuse or polysynaptic type of bipolar cell. One of these, the *d* bipolar, corresponds to Cajal's "rod bipolar," but it is found to be related synaptically to cones as well as rods, so that Cajal's terminology is not altogether apt. Attention is also drawn to the fact that not only is the dendritic tuft of each *d* bipolar related to a compact group of photoreceptors, but the tufts spread over the territories belonging to those of adjoining bipolar cells of all varieties, both diffuse and individual. The individual bipolar may be appropriately termed a "cone bipolar," since this variety is related to cones alone. As a rule there is no reciprocal overlapping between the dendrites of adjoining cone bipolars, particularly in the central region of the retina, and here most of them are related individually to single cones. In the more peripheral parts of the retina, however, a limited degree of overlapping may occur. A consideration of the disposition of the diffuse bipolar cells indicates that even at this neural

level in the visual path (and still more so at the ganglion cell level) the two retinal mechanisms related respectively to photopic and scotopic vision are by no means completely separated from each other. Another point which emerges from this histological study is that, although it is not possible by Golgi impregnation to demonstrate more than a single morphological type of cone, the several varieties of bipolar cells may constitute the analysers postulated by the three-component hypothesis of colour vision of Young, Helmholtz and Maxwell.

As with the bipolar cells, Poliak found in the higher primates two groups of ganglion cells, the diffuse and the individual. Each individual ganglion cell (also called a "midget ganglion cell" because of its small size) effects a synaptic contact with a single individual or cone bipolar cell, and is therefore related to a single cone. Thus, as Poliak emphasizes, there is indeed an anatomical basis for a one-to-one relationship between the neurones of different levels in the retina. However, it seems that some individual ganglion cells may at the same time be in synaptic contact with bipolar cells of the diffuse type. The diffuse ganglion cells have relatively long or numerous dendrites, with a considerable reciprocal overlapping of the territories of adjacent cells.

Horizontal cells are present in the inner nuclear layer and are most numerous in the macula and fovea. Their axones are disposed in no particular direction and each cell brings into reciprocal relationship one group of cones (up to 15 in the macula) with another and larger group of cones and possibly also with a group of rods. The "amacrine" cells are difficult to evaluate, for the term has been applied to glial as well as to nervous elements. Some appear to be associational cells with a horizontal axone running in the inner molecular layer for some distance; others appear to be merely ganglion cells displaced into the inner nuclear layer.

In relation to the purely histological evidence for a point-to-point projection system from the retina to the lower visual centres, may be mentioned the oscillographic observations of Hartline (43). This author investigated the receptive fields of individual optic fibres in certain cold-blooded vertebrates (frog and alligator) by recording the action potentials in single fibres in response to the illumination of various parts of the retina. It was concluded from these experiments that the receptive field of an optic nerve fibre covers an area much greater than that occupied by a single rod or cone; that a retinal ganglion cell can therefore receive excitatory influences over many convergent pathways; and that the axone of a ganglion cell is simply the final common path for nervous activity originating in many sensory elements. However, though this may be the case with many optic fibres (as is certainly affirmed by the histological observations of Poliak), it does not seem possible on the basis of Hartline's work to exclude other types of fibre with much more restricted receptive fields. In any case, of course, Hartline's studies were not concerned directly with the mammalian retina.

Optic nerve. Contrary to some statements, observations of Bruesch and Arey (18) have shown that in most mammals including man and other primates all the fibres of the optic nerve are myelinated. In some lower mammals, however, a considerable proportion may remain unmyelinated (e.g., 33 per cent

in the opossum and 44 per cent in the bat). These authors have also shown that the number of fibres in the optic nerve shows considerable variation in mammals, partly (but by no means consistently) related to the size of the eye. The following computations were made:

| | |
|----------------------------------|-------------------|
| Opossum..... | 82,000 |
| Bat (species not indicated)..... | 7,000 |
| Dog..... | 146,000 |
| Cat..... | 118,000 |
| Rabbit..... | 265,000 |
| Guinea pig..... | 126,000 |
| Monkey..... | 1,208,000 |
| Man..... | 565,000-1,140,000 |

Perhaps the most striking feature of this table is the fact that in the rabbit (an animal in which the panoramic type of vision predominates) the optic nerve fibres are more than twice as many as those of a cat (an animal in which visual acuity is commonly regarded as relatively well developed and in which the optic nerve is actually larger in diameter). It is also somewhat surprising to find that a monkey has more optic nerve fibres than man; on the other hand, it is a fact that the lower and higher visual centres of the brain of some species of monkey do show a distinctly higher degree of structural differentiation. The numerical relation of optic nerve fibres to retinal ganglion cells has been recently studied by Arey (2). Whereas previous observations had suggested that the number of ganglion cells is much in excess of the optic fibres (indicating that many of the ganglion cells do not send processes back to the brain), Arey found that in the dog there is good reason to believe that a simple 1:1 ratio exists. It is probable, indeed, that in earlier work the numerous glial cells of the ganglion cell layer of the retina had not been excluded from ganglion cell counts.

The fibres of the optic nerve show a tendency to a topographical grouping in relation to the retinal site of their origin, but it appears that this localization is not very sharply defined. Brouwer and Zeeman (17), on the basis of Marchi preparations, found in the monkey that the fibres from the upper half of the retina are situated above those from the lower, and that the temporal fibres lie lateral and the nasal medial. On the other hand, they concluded that the macular fibres do not show a very exact localization; near the eye they occupy a lateral position in the optic nerve, but as they approach the chiasma they come to be more centrally placed. It is evident, therefore, that some regrouping of fibres occurs in the antero-posterior course of the optic nerve.

The optic nerve fibres also show a differentiation according to size and rate of conduction. Curiously enough, the anatomical details of this differentiation have hitherto received very little attention, though appropriately stained sections of the optic nerve demonstrate clearly enough that there is a considerable range in the calibre of the fibres, and this range apparently varies in different species of mammal. The problem has however been approached from the electro-physiological side by Bishop (10). This author found evidence for the existence in the frog's optic nerve of three rather distinct groups of fibre, differing in conduction rate, threshold to electrical stimulation, and other physio-

logical properties. In the rabbit's optic nerve oscillographic records were found to be similar to those of the frog, except that one wave (the *C* wave) appeared to be lacking or else extremely low. The first group of fibres in the optic nerve corresponds in conduction rate not to the first group in most general sensory nerves, but to a slower element. In the rabbit, for example, the rate is somewhat faster than that characteristic of the sensory B group of the saphenous nerve, which mediates pain, temperature and vaso-motor impulses. The second group does not seem to correspond with any well-defined group in other types of sensory nerve. Lastly, it is supposed that the third group (in the frog) may consist of efferent fibres. The significance of these different types of fibre in the optic nerve is unknown, except that (in mammals) the larger fibres are mainly destined for the lateral geniculate body and the finer fibres for the mid-brain. Bishop remarks that by analogy with the sensory nerves "one might anticipate that the larger fibres of the optic nerve would mediate that aspect of vision concerned with spatial discrimination or form, while the smaller fibres would be concerned with the quantitative factor of intensity."

The presence of centrifugal fibres in the optic nerve, passing to the retina, has been a source of dispute in the past. Several authorities have expressed doubt as to whether such fibres exist in mammals and this doubt now seems to be fully confirmed by the study of the fibre content of optic nerves after enucleation of the eye. Bodian (13), for example, reports that, in the opossum, no normal nerve fibres are to be found in reduced silver preparations of optic nerves 10 weeks after removal of one or both eyes.

Previous investigations seem to have established the fact that in certain lower vertebrates efferent fibres to the retina do exist in the optic nerve. For example, Arey (1) brought forward experimental evidence to show that in *Ameiurus* there are functional efferent nerves which in some manner control the photomechanical responses of the rods, cones and retinal pigment. He suggested that the efferent impulses do not directly stimulate the motility of the retinal elements, but that they have an indirect action by counteracting the tonic inhibition exerted by the ciliary nerves. However, in certain other fishes, and also in frogs, no such function could be demonstrated, and it remains possible, therefore, that the mechanism is peculiar to *Ameiurus*.

Optic chiasma. It has not infrequently been stated that a partial decussation of the retinal fibres in the chiasma only occurs in man and in the higher mammals—in lower mammals the decussation was presumed to be complete. It is probably true to say, however, that in no mammal is this the case. In the lowly opossum, for example, as many as one-fifth of the retinal fibres remain uncrossed (Bodian, 13), and in the ferret about one-third (Jefferson, 47). It has also been supposed that the development of only a partial decussation of the optic nerves is related to the development of a fovea centralis in the retina, and to the development of a precise type of conjugate movement. This again seems to have been a misconception, for Detwiler (33) has pointed out that a fovea is found in fishes and Lacertilia in which the decussation of retinal fibres is complete, and, moreover, the complete decussation in the chameleon is hardly consonant with the

suggestion that a partial crossing is a necessary condition for a wider range and a greater exactitude in conjugate movements of the eye.

The proportion of optic fibres which cross in the optic chiasma in higher mammals has not been established by exact computational methods. It is known from experimental evidence that in monkeys all retinal fibres from the temporal side of a vertical axis passing through the centre of the macula pass to the ipsilateral optic tract, while those from the nasal side undergo a decussation. Indirect observations on the monkey (based on the relative number of cells in the geniculate body receiving, respectively, crossed and uncrossed fibres) have suggested that in this animal (so far as the geniculate body is concerned) about 40 per cent of the fibres in each optic nerve remain uncrossed (Clark, 28).

The intimate topographical relation between the optic chiasma and certain hypothalamic commissures in this region make it very difficult in normal preparations to decipher the precise course of the retinal fibres here. From time to time statements have been made with reference to optic fibres that are supposed to leave the chiasma and terminate in the grey matter of the hypothalamus. Experimental studies, however, have discounted such a conclusion. In a critical examination of the optic tract system of the ferret, Jefferson (47) showed that it is very easy to misinterpret the appearance of normal Weigert and silver preparations. For one thing, the fibres of the supra-optic commissures (which probably have nothing to do with visual functions) are not always distinguishable from retinal fibres. Also, in the chiasma the decussating fibres may run an aberrant course which can be very misleading to the casual observer. Some fibres, for example, may run up from the chiasma towards the wall of the 3rd ventricle and then loop down again to rejoin the optic tract on the opposite side. It seems certain that such aberrant fibres have been mistaken in the past for terminal optic fibres related to the hypothalamus, simply because they have not been traced in appropriately stained material by the study of serial sections. The discrete median "dorsale hypothalamische Wurzel" of the optic tract described by Frey (36) as entering the tuber cinereum from the dorsal and caudal aspect of the chiasma is evidently based upon an error of interpretation of a different category, for he seems to have mistaken the appearance of a tangential section of the dorsal convexity of the chiasma for a median tract.

Optic tract. All the experimental evidence at present available goes to show that the homolateral and heterolateral fibres from corresponding areas of the retinae are intimately and evenly mingled with each other in the optic tract. Even in man and other higher primates there is no indication of a stratification such as is found in the terminations of these sets of fibres in the main lower visual centre—the lateral geniculate body. Thus, some weeks after the section of one optic nerve in a monkey, the remaining normal fibres are found to be disposed perfectly evenly throughout the optic tracts (Clark, 28). It follows, therefore, that the crossed and uncrossed fibres only become disentangled and separated from each other inside the geniculate body. On the other hand, it has been established for some time that, at least in the primates, the fibres from the different quadrants of each retina retain a fairly definite segregation in the

optic tract, in the sense that those from the upper quadrants are disposed dorsally, and those from the lower quadrants ventrally. The macular fibres occupy the central part of the tract, extending over a fairly wide area dorso-laterally.

Accessory optic tracts. Besides the main optic tract which proceeds back from the chiasma to the lateral geniculate body and the mid-brain, two accessory tracts have been described which, leaving the main tract, terminate in the region of the subthalamus. The anterior accessory optic tract (of Bochenek) is commonly described as penetrating the cerebral peduncle to end in the subthalamic nucleus (*Corpus Luysii*), while some of its fibres may reach the same nucleus more directly by passing over the dorsal surface of the cerebral peduncle. The presence of this tract was affirmed in Marchi preparations of the rabbit's brain by Pavlow (71) and Loepp (56). However, there remains grave doubt whether a tract with such connexions really exists, for other interpretations of this bundle of fibres have been put forward. For example, Kosaka and Hiraiwa (49) believed it to be merely an aberrant bundle which subsequently rejoins the main optic tract. Lashley (51) identified the tract in the rat's brain, but found that it divides into two branches, of which one rejoins the main optic tract while the other joins the posterior accessory optic tract. The previous work of Overbosch (69) had also suggested that the anterior tract might be simply an aberrant part of the posterior tract. The apparent absence of an anterior accessory optic tract has been recorded in the opossum (Bodian, 13) in the cat (Barris *et al.*, 7) and in the ferret (Jefferson, 47). Indeed, whatever may be the case in the rat and rabbit, the opinion is expressed by Barris *et al.* that the tract does not exist in higher mammals. In a more recent study, Gillilan (37) affirms the existence of the tract (as well as its termination in the subthalamic nucleus) in bats, insectivores and rodents, this conclusion being partly based on the study of Marchi preparations from experimental material. Probably, however, before these conclusions are finally accepted in the face of the negative observations recorded by many careful workers, it will be necessary to employ an experimental technique which is more refined than the Marchi method. Incidentally, it may be noted that the fibres of the so-called anterior accessory optic tract are extremely fine, so that it is by no means easy to follow them accurately with Marchi staining.

The posterior accessory optic tract is described as leaving the main optic tract close to the ventral pole of the lateral geniculate body, crossing obliquely over the cerebral peduncle (forming here the *tractus peduncularis transversus*), and entering the interpeduncular space. Here it terminates in a collection of cells, the nucleus opticus tegmenti, which is interposed between the mammillary body and the cerebral peduncle, and also (probably) in the medial extremity of the substantia nigra. There can be little doubt that the posterior accessory optic tract exists in some lower mammals. Since it was first described by Gudden (41), its existence has been confirmed experimentally in the rabbit by Pavlow (71), Loepp (56), in the guinea pig by Wallenberg (86), Castaldi (20) and Frey (36), in the rat by Clark (24), Lashley (51), Chang (21) and Tsang (82), in the opossum by Bodian (13), and in the phalanger by Packer (70). But there remains con-

siderable doubt whether the tract is present in the brain of higher mammals. Thus Barris *et al.* (7) were unable to find it in the cat, or Jefferson (47) in the ferret. On the other hand, Gillilan (37) has more recently reached the conclusion that it is present in the cat and the monkey, but it must be admitted that the evidence of this author as presented is perhaps not altogether convincing. Lastly, it is well to point out that statements made in the past by some comparative neurologists on the course and distribution of the accessory optic tracts have evidently been based entirely on the examination of normal histological material. Such material, however, is readily liable to misinterpretation, for it does not permit of the assumption that fibres seen in the position of the so-called accessory optic tracts are really retinal in their origin.

The functional significance of the accessory optic tracts when they are present (e.g., in rodents) is quite unknown. Lashley (53) concluded from his experiments that they are not adequate for the formation of visual habits based on brightness discrimination. Attempts were made by Clark, McKeown and Zuckerman (31) to determine whether they might be concerned with the gonadal response shown to retinal stimulation in ferrets. It was found that bilateral section of the optic tracts at the ventral pole of the lateral geniculate body made no difference to this response, which seemed to indicate that it might be mediated by the accessory tracts. However, the evidence was inconclusive, for careful examination of serial sections showed that in all the experiments a very few optic fibres (passing either to the lateral geniculate body or to the mid-brain) had escaped injury on one side. This problem, therefore, requires a re-investigation.

The tectal connexions of the optic tract. Most of the retinal fibres which reach the roof of the mid-brain (at least in most mammals) end in the superior colliculus. Marchi preparations following section of the optic nerve have shown that these collicular fibres can be followed into the layer of white matter which is termed the stratum opticum. Hereafter the fibres in general lose their myelin sheaths, so that the actual site of their terminal arborization cannot be followed with certainty either in Weigert preparations, or by the Marchi technique in which degenerating myelinated fibres are stained differentially with osmic acid. By the latter method, indeed, some observers have followed mesencephalic fibres of the optic tract from the stratum opticum into a layer of cells lying immediately superficial to it, the stratum griseum superficiale, and from silver-impregnated sections of normal material a similar conclusion can be drawn. Further, it has been shown by Tsang (82) that the cells of the stratum undergo a pronounced degree of atrophy several months after section of the optic nerves in young rats. The precise site of termination of optic fibres in the tectum has recently been established by studying silver-impregnated material from animals in which one optic nerve had been sectioned 48 and 72 hours before death (Jefferson, 47). In these sections it was found that the terminal ramifications of the mesencephalic retinal fibres had undergone a very distinct fragmentation and granulation, and these degenerative changes were found to extend not only throughout the stratum griseum superficiale but also in the most superficial zone of the superior colliculus, i.e., the stratum zonale. It may now be accepted,

therefore, that retinal fibres run into the superior colliculus to form a layer of white matter, the stratum opticum, and terminate in all that part of the colliculus which lies superficial to this layer.

The localization of retinal fibres in the superior colliculus was studied in the rabbit by Overbosch (69) and in the rat by Lashley (51). In these animals the localization is of a fairly precise nature, to the extent that the lower temporal fibres of the retina are projected on to the antero-medial quadrant of the colliculus, the upper temporal to the antero-lateral quadrant, the inferior nasal to the postero-medial, and the superior nasal to the postero-lateral. Bodian (13) has reported a similar localization in the opossum. The course and ending of the mesencephalic fibres suggest that in the monkey also there is a localization of the different parts of the retina over the surface of the colliculus. It seems that in many mammals few if any uncrossed fibres from the retina reach the superior colliculus or, indeed, any part of the tectum. Certainly this appears to be the case with the rat, rabbit and ferret, and, indeed, such a conclusion is consonant with the conception that the mesencephalic tectum is the most primitive of the lower visual centres and that in lower vertebrates the decussation of optic fibres in the chiasma is primitively complete. On the other hand a few retinal fibres have been described as passing to the homolateral colliculus by some observers, e.g., Barris, Ingram and Ranson (7) in the cat and Bodian (13) in the opossum, but these are far less numerous than the fibres which pass to the opposite colliculus.

In their experiments on the monkey Brouwer and Zeeman (17) had concluded on the basis of Marchi material that no fibres from the macula reach the superior colliculus, and this was rather a surprising result since it is supposed that pupillary reactions are most lively when light falls on the macula. It was indeed concluded that if fibres concerned in the reflex movements of the pupil take origin in the macula they must be unmyelinated (for such fibres would not of course be evident in Marchi preparations). However, as we have already noted, the observations of Bruesch and Arey have demonstrated that in the monkey there are no unmyelinated fibres in the optic nerves. In recent years the explanation of this discrepancy has become apparent. For a long time comparative anatomists have described in the brain of lower mammals a rather well-circumscribed oval group of small cells situated at the dorsal surface of the tecto-thalamic junction, immediately under cover of the anterior and lateral margin of the superior colliculus. This group of cells is now termed the pretectal nucleus. By some authors the element has been regarded as the homologue of the pulvinar in the primates, but this identification is certainly erroneous, for the pulvinar is nothing more than the posterior part of the lateral nucleus of the thalamus which, in higher mammals, has become pushed backwards to form a prominent eminence by the expansion of the main part of the lateral nucleus which lies in front of it (Clark, 23). Lashley (51), in Marchi material, found degeneration within the pretectal nucleus following section of an optic nerve, and convincing evidence has since been adduced to show that the pretectal nucleus is an essential element in the pathway of the reflex constriction of the pupil.

The course of the pupillo-constrictor fibres has been followed in the cat by Hare, Magoun and Ranson (42) by noting the pupillary reaction which occurs on the electrical stimulation of successive points along the optic tract with a stereotaxic apparatus. By previous sectioning of the optic nerves, allowing time for the retinal fibres to undergo degeneration, they have shown that the pupillo-constrictor fibres have their first synapse in the pretectal nucleus. From here the impulses are relayed by fibres which mostly cross in the posterior commissure to the oculomotor nucleus of the opposite side, though some pass to the ipsilateral nucleus. Pupillo-constrictor fibres of cortical origin extend from a cortical area adjacent to the visual cortex directly to the pretectal region. Hence lesions of the optic tract may abolish pupillary reaction to light while maintaining the integrity of the cortical conveyance reaction. In the monkey, the same disposition was found. Light reflex fibres pass toward the superior colliculus. They do not enter the latter, however, but turn rostrally and medially into the pretectal region. Thence the path passes to the oculomotor nucleus by fibres which may decussate in the posterior commissure and also ventral to the cerebral aqueduct.

If the light reflex of the pupil is mediated by the pretectal nucleus which lies under cover of the margin of the superior colliculus, it remains to consider what functions are subserved by the superior colliculus itself in virtue of the termination in its superficial layers of retinal fibres. In the monkey it is commonly stated that relatively few optic fibres reach the superior colliculus and that in man the retinal connexions are even more insignificant. However, it seems that such statements do not depend on any method of direct computation but are mainly based on the general impression gained by the study of sections of Marchi material. Marchi sections, however, are easily open to misinterpretation in this kind of problem. For one thing, the optic fibres which reach the mid-brain are more finely medullated than those which terminate in the lateral geniculate body; thus the Marchi staining will be relatively much less intense in the mesencephalic fibres, suggesting to the uncritical eye that they are few in number. In lower mammals large numbers of retinal fibres certainly end in the colliculus and in correlation with this it has been shown experimentally that in these animals the superior colliculus provides an integrating mechanism of considerable complexity in relation to visual impulses. It has been shown, for example, that rodents with no visual cortex at all can discriminate light intensity and can also judge the position and distance of an object (Ten Cate and van Herk, 81; Lashley, 50). Under similar conditions dogs can also retain their powers of discrimination for different intensities of light, though in these animals the recognition of the position and distance of an object is a cortical function (Marquis and Hilyard, 59; Marquis, 57). Even in monkeys a conditioned response which has been established to a light stimulus may be retained after the entire removal of the visual cortex (Marquis and Hilyard, 60), whence it may be assumed that this function is mediated by the mid-brain. On the other hand, destruction of the visual cortex in man leads to complete and permanent blindness. Marquis (58) has drawn attention to these differences in the results

following removal of the visual cortex in different species, and has shown that it represents a phylogenetic sequence in the gradual "corticalization" of visual functions in the brain.

The lateral geniculate body. The lateral geniculate body is to be regarded as one of the sensory nuclei of the thalamus, and in lower mammals it may be only imperfectly demarcated from the main ventral nucleus of the thalamus in which fibres of the fillet system terminate. In primitive mammals, also, the visual fibres which end in the lateral geniculate body are probably entirely collaterals of the main optic tract fibres which are passing by on their way to the mid-brain. In other words, primitively the lateral geniculate body appears to be functionally subordinate to the tectum as a visual centre. If mammals are arranged in a phylogenetic series it is possible to demonstrate that, in an ascending scale, proportionately more and more direct optic fibres end in the lateral geniculate body, and, since the latter is essentially a relay station for the projection of retinal impulses on to the cortex, this is an anatomical reflexion of the increasing dominance of the cortical control of visual functions in the evolutionary series.

If the lateral geniculate body of a lower mammal (e.g., a rabbit) is viewed in a transverse section of the thalamus, it appears to consist of two parts—a larger portion with relatively large cells, the dorsal nucleus of the lateral geniculate body, and a ventral segment containing small cells, the so-called ventral nucleus. In Weigert and silver sections, bundles of the optic tract can be seen to run through the ventral nucleus on their way to the dorsal nucleus, and a number of fibres appear to end in the former. Indeed, since Cajal (19) described and figured collaterals from the optic tract fibres ending in the ventral nucleus, this element has been for a long time widely accepted as one of the lower visual centres. Moreover, it has been regarded as a more primitive diencephalic visual centre than the dorsal nucleus, for it is but a lateral extension of the lower functional levels of the subthalamus, and it shows a gradual retrogression in an ascending phylogenetic series. However, doubt has been expressed in recent years as to whether the ventral nucleus in reality has anything to do with vision. The difficulty of arriving at a decision depends on the fact that in this region the fibres of the optic tract are intermingled to a considerable extent with other fibre systems, so that, however persuasive the appearance of a normal Weigert or silver preparation may be, it is not possible with such material to affirm with certainty that *retinal* fibres end in the ventral nucleus of the lateral geniculate body. Certainly a large proportion of the optic tract fibres here are merely fibres of passage directed towards the dorsal nucleus. The study of Marchi degeneration after section of one optic nerve leads to negative results in this problem, for the osmic staining appears to be confined to the fasciculi of fibres of passage, while the ground-substance of the nucleus remains free of deposit. This is in marked contrast with the dorsal nucleus of the lateral geniculate body, the ground-substance of which is richly sprinkled with black osmic granules in similar experiments. There remains the possibility, however, that fine unmyelinated collaterals of the optic fibres (which would not be demonstrable by the Marchi technique) terminate in the ventral

nucleus. On the basis of Golgi material of the cat's brain, O'Leary (66) has recently described very fine fibres which leave the position of the optic tract to end in the nucleus, but, here again, it has yet to be proved that these fine fibres have their origin in the retina. The study of silver impregnated material in which the optic tract has undergone complete degeneration after section of the optic nerves provides no further positive evidence, for it has been found that in the ferret such material shows no diminution in the fine neuropil pervading the ventral nucleus as compared with the normal side. Lastly it has been observed by Tsang (82) that, after unilateral or bilateral extirpation of the eyes in rats, there is never atrophy of the cells of the ventral nucleus of the lateral geniculate body, however long the period of degeneration. On the other hand, after similar operative procedures the cells of the dorsal (or main) nucleus become shrunken, rounded and embryonic in form, and with fewer processes than normal.

The main part of the lateral geniculate body¹ (the dorsal nucleus as it is commonly termed by comparative anatomists) is by far the most important of the lower visual centres in higher mammals, and particularly in man. In a phylogenetic series of mammals it undergoes an increasing cyto-architectural differentiation, and also shows a progressive tendency to become clearly and sharply laminated. Such a laminar arrangement of the geniculate cells may be seen in an incipient form even in the brains of primitive mammals; in higher mammals it becomes very distinct, and it is now known that this lamination is primarily related to the termination of crossed and uncrossed fibres. But the number of cell-layers varies in different groups. In the cat, for example, there are three main laminae, and of these the middle lamina receives uncrossed retinal fibres while the other two are related to crossed fibres. In all the higher primates (including man, apes and Old World monkeys) there are six laminae, three of which are connected with each retina.

The precise mode of termination of retinal fibres in the lateral geniculate body is a matter of considerable interest from the point of view of visual physiology. First of all it may be mentioned that, at least in lower mammals (and possibly in man also), mesencephalic fibres of the optic tract may give off collaterals which terminate in the lateral geniculate body. This is the case, for example, in the cat, and it has been emphasized that it is of some functional significance that one and the same fibre from the retina may mediate impulses to the tectum of the mid-brain, and also the visual cortex by way of the lateral geniculate body (Barris, Ingram and Ranson, 7).

The earlier work of Brouwer (15) and Brouwer and Zeeman (17) had shown that a localization of quite a precise type is present in the lateral geniculate body. In the rabbit the localization is such that the upper half of the retina projects on to the lower half of the lateral geniculate body and *vice versa*. In the monkey, following the rotation of the lateral geniculate body which has occurred during its development, the projection is rather different, for now the fibres from the upper half of the retina end in the medial half of the nucleus, while those from

¹ Hereafter the term *lateral geniculate body* will be used entirely in reference to the main or dorsal nucleus.

the lower half of the retina terminate laterally. In the monkey, also, the macula has quite a definite representation of its own, and this occupies a relatively large area which appears as a median sector of the geniculate body.

In 1934 Clark and Penman (30) carried out experiments on monkeys similar to those of Brouwer and Zéeman (by the production of small lesions in different parts of the retina), but using instead of the Marchi technique a more precise method for localizing in the lateral geniculate body the site of termination of the retinal fibres which had been involved in the lesions. This method depends on the fact that a very marked transneuronal degeneration occurs in the lateral geniculate body of monkeys after the interruption of optic fibres. The reason for this type of degeneration in this particular nucleus is not clear, unless indeed it is an indication of the extreme specificity of function of the cells of the geniculate body to the extent that they undergo rapid atrophy if they are not activated by the impulses which normally have their origin in the retina. In some lower mammals which have been investigated by the present writer (e.g., the rat and ferret) such distinct transneuronal degeneration does not occur, and in the cat it seems to be relatively slight, the changes in the cells "amounting to no more than a diminution of the amount of cytoplasm" (Barris, 5). In the monkey (and in man also) it is so marked as to allow a very precise determination of the position of any group of cells in the geniculate body which receives fibres from a small area of the retina.

By making use of this phenomenon of transneuronal degeneration it was possible to demonstrate that the retinal projection on to the lateral geniculate body in monkeys is even more precise than had been supposed. Indeed, practically speaking, the projection approximates to a point-to-point representation, for it is possible to map out the whole of the lateral geniculate body in terms of small and discrete retinal areas. The macular area of the retina was found to be limited to the caudal two-thirds of the nucleus, occupying a median sector which widens in a caudal direction at the posterior extremity where it covers the whole width of the nucleus. Various observations have also indicated that the localization of the macular and peripheral areas of the retina in the nucleus in man, anthropoid apes and monkeys is of an equivalent order (Balado and Franke, 3, 4; Brouwer, 16; Poliak and Hayashi, 76). The transneuronal experiments also demonstrated some interesting features with regard to the termination of crossed and uncrossed fibres in the geniculate body, and in order to give an account of this it is necessary to refer to the structure of the geniculate nucleus in man and monkey.

In the higher primates the lateral geniculate body is composed of six distinct and clearly demarcated cell laminae, separated by intervening layers of white matter. The two most superficial laminae are composed of relatively large cells, and the remaining four of closely-packed smaller or medium-sized cells. For convenience of description the layers may be numbered 1 to 6 from the surface inwards. Many years ago Minkowski (64) found that in a monkey in which one optic nerve had been sectioned some time before death, the lateral geniculate body of the opposite side showed a pronounced atrophy of the cells forming

layers 1, 4 and 6, while on the same side layers 2, 3 and 5 had been similarly involved. In other words, it appeared that crossed fibres of the optic tract terminate in one set of cell laminae (1, 4 and 6) while the uncrossed fibres end in the other set (2, 3 and 5).

Clark and Penman found in their experiments that the smallest retinal lesion which produces detectable changes in the lateral geniculate body nerve cells always leads to a zone of atrophy in the form of a band affecting all three of the corresponding cell laminae of the lateral geniculate body. In other words, this is the receptive unit of the lateral geniculate body in respect of each retina; these units altogether may be described as a series of narrow bands radiating from the region of the hilum towards the convex periphery of the nucleus, involving the three laminae related to the retina of one or other eye.

This arrangement is susceptible of two possible interpretations. Either 1, each individual fibre from the retina (the axonal process of a single ganglion cell) terminates by a series of arborizations about cells in all the three laminae concerned, or 2, the conducting unit of the optic nerve in respect of the lateral geniculate body is a unit of three fibres each passing to a separate lamina. The first alternative is almost certainly incorrect, for a careful study of silver-impregnated material fails to show any indication of the division of individual optic fibres, either in their course through the optic nerve and tract, or at their point of entry into the lateral geniculate body. The second alternative, it has been suggested (Clark, 27), may have a relation to the Young-Helmholtz theory of colour vision, for this theory involved the assumption of three types of sensory receptor in the retina and three sets of optic nerve fibres connecting them with the brain. In other words, it seems possible that the three laminae of the geniculate body related to each retina may be concerned with the three fundamental colour sensations postulated by the trichromatic theory of colour vision—red, green and violet.

Such a conclusion, of course, must remain entirely speculative in the absence of direct evidence. It may be noted that a recent study of the lateral geniculate body in the New World monkeys (Clark, 29) has failed to show any correlation between the degree of visible lamination and the differentiation of colour-vision as determined experimentally. It is true that in *Cebus*, which according to the evidence recorded by Grether (40) possesses dichromatic vision, the lamination is by no means so clear-cut as it is in *Macaca*. On the other hand, in the spider monkey (in which it appears that colour discrimination is fully equal to that of the Old World monkeys) the small-celled element of the lateral geniculate body shows no visible lamination. It is pointed out, however, that there may be a functional lamination which is not visibly manifest in the nucleus, and the existence of which could only be determined by the study of transneuronal degeneration following the section of one optic nerve.

It is of some importance to determine whether any mechanism exists in the lateral geniculate body which could provide the anatomical basis for a fusion at this level of crossed and uncrossed retinal impulses. It is well known that

small lesions in the lateral geniculate body lead to small, bilateral scotomata. But it is hardly possible that even the smallest lesions which can lead to a demonstrable scotoma will be confined to one cell-lamina only, and, even were this the case, it must inevitably involve optic fibres which penetrate the lamina to reach the next adjacent lamina. Henschen (45), on the basis of Golgi studies of the human geniculate body, concluded that intercalated elements are present through which the several cellular laminae are brought into functional relation with each other. However, Taboada (79) maintains that the diverse types of cell described by Henschen really represent different varieties of a single type, the differences in appearance being entirely due to the topographical position in which each cell finds itself. The same author has also established that, in the monkey, the dendritic processes of the geniculate cells are confined in their distribution to the individual lamina in which the cells are situated. In spite of the pronouncements to be found in the earlier work of neurohistologists, the precision with which transneuronal atrophy following section of one optic nerve is limited to one set of laminae only suggests a very high degree of functional independence. Further, the fact that complete ablation of the visual cortex leads to a total atrophy of all the cells of the large- and small-celled laminae of the lateral geniculate body seems to decide finally against the existence in these laminae of any intercalated neuronal elements whose functions might be presumed to link them up with each other. In other words, it is highly probable on anatomical grounds alone that crossed and uncrossed visual impressions remain isolated from each other in the lateral geniculate body of higher mammals, so that fusion can only occur in the visual cortex. A study of the electrical activity following optic nerve stimuli provides support for this conclusion, for, leading off from the geniculate body of one side (in the cat) while stimulating simultaneously or in succession both optic nerves with single shocks, Bishop and O'Leary (11) found no evidence of any phenomena of interference or facilitation which might suggest fusion of retinal images at the geniculate level. On the other hand, it had previously been reported by Marshall and Talbot (61) that facilitation can occur at the cortical level. Further findings from the oscillographic studies of Bishop and O'Leary may be mentioned here. In correlation with the histological evidence that, generally speaking, the finer fibres of the optic tract run to the mid-brain, while the coarser fibres end in the geniculate body, it was found that impulses reach the latter more rapidly. Indeed, it appears that, allowing for the conduction and synapse time in the pathways to the optic centres, then of two impulses started in parallel fibres of the optic nerve at the retina, the one passing to the cortex can be relayed down to the superior colliculus before the other has reached the colliculus directly. According to the authors, it would then be possible on physiological grounds for an animal to recognize a sensory stimulus in a shorter time than it could respond to it reflexly, i.e., there would be time for a message from the cortex to reach the reflex centres for regulation of the response before the reflex centres were activated directly by the same stimulus. The experiments further suggested that the majority of the fibres which activate the cortex do not activate the superior colliculus, and *vice versa*.

It is of considerable morphological interest to note again that, while the crossed and uncrossed retinal fibres terminate in different sets of laminae in the lateral geniculate body, they show no tendency to assume a laminar arrangement as they approach the latter in the optic tract. On the contrary, in the tract the crossed and uncrossed fibres are intimately intermingled. It thus appears that there is no question of the laminar arrangement of cells being secondarily imposed on the lateral geniculate body by the disposition in which the retinal fibres find themselves on their arrival at the end of the optic tract. On the contrary, the optic tract fibres penetrate the anterior and ventral aspect of the geniculate body and may pass through several layers of cells before they reach their own particular lamina.

The relation of the laminae in the lateral geniculate body to crossed and uncrossed optic fibres has been shown to be the same in man as in the monkey (Clark, 25; Hechst, 44; Barris, 5). In the cat, the nucleus consists of three main cellular laminae (of which the most superficial is sometimes subdivided topographically into two). Barris (5) has confirmed an earlier observation of Minkowski (63) that crossed retinal fibres terminate in the most superficial and the deepest of these layers, while the intermediate lamina receives uncrossed fibres.

While it is certain that in mammals such as man, monkeys and cats in which the lateral geniculate body is distinctly laminated the crossed and uncrossed retinal fibres end in different layers of cells (though always in those parts of the layers which lie immediately adjacent to each other), it is usually not possible to determine the relation of the termination of crossed and uncrossed fibres in more lowly types of mammal, for in these the method of study by transneuronal degeneration is not sufficiently precise to allow of its application. It is known however that in the rabbit (Brouwer and Zeeman, 17), rat (Lashley, 51) and opossum (Bodian, 13) the crossed and uncrossed fibres from corresponding points in the two retinae always end in the same region of the lateral geniculate body (though whether in connexion with the same or immediately adjacent cells is not known). However, a recent experimental study of the geniculate body in the phalanger (Packer, 70) has produced rather surprising evidence that in this marsupial crossed and uncrossed fibres end in relation to alternating laminae of cells, though the laminae are by no means as distinct as they are in the higher eutherian mammals.

The mode of termination of optic fibres in the lateral geniculate body. Earlier studies of Kölliker (48), Tello (80) and Cajal (19), based on Golgi preparations of fetal and immature material, had shown that in the mouse or cat each optic fibre breaks up into a complicated brush of fine terminals enclosing a nest of cells estimated at about 6-8 in number. More recent Golgi studies by O'Leary (66) on the cat have confirmed and considerably extended these observations. He found that the thick axones of the optic tract divide close to the main nucleus of the lateral geniculate body into thick and thin branches. Of these the former actually penetrate into the nucleus, and the terminal ramifications of each one are confined to a single cell lamina and the interlaminar margin

in proximity to it. Within each cell lamina the terminal arborizations overlap each other so that each principal cell makes synaptic contacts with the terminals of several different optic tract fibres. O'Leary also describes in the cat short-axone cells which are distributed throughout the laminae (and distinct from the principal cells whose axones proceed to the visual cortex). The axones of the principal cells may give off a few slender collaterals in the layer to which the cell belongs, but this appears to be "the exception rather than the rule." This last observation, it may be noted, is perhaps in some contradistinction to the previous observations of Tello, who specifically calls attention to the presence of such collaterals. O'Leary, however, believes that his histological evidence is in accordance with the electro-physiological observations that self-reactivation is not an important phenomenon in the lateral geniculate body of the cat, for these observations have demonstrated that fast conduction occurs through the nucleus with no sequelae of sustained activity. He believes, moreover, that the short-axone cells cannot play the rôle of interneurons in the lateral geniculate body, but that they may act as synchronizers for groups of principal cells. An experimental study by Glees (38) of the cat's geniculate body, with the application of refined silver impregnation methods, has demonstrated that, in the mature animal at least, the optic fibres end in the nucleus in special end-formations in the shape of fine terminal rings (entirely similar to the terminal boutons which have been demonstrated in relation to the motor cells of the spinal cord and elsewhere). Moreover, these boutons, as well as the terminal branching of the optic fibres, undergo characteristic degenerative changes after section of the optic nerve. It was estimated that the number of synaptic contacts of optic terminals established with one principal cell amounts to about forty and these contacts are related to the dendrites as well as the cell body. The axo-dendritic contacts, however, are more numerous than the axo-somatic. Lastly, this histological evidence suggested that each optic fibre covers with its terminal branches an area containing about ten cells, and that there is probably an extensive overlap. Similar experimental studies of the geniculate body of the macaque monkey (with the use of the same silver technique) have revealed a rather different state of affairs (Glees and Clark, 39). Here terminal boutons are also present, but in much fewer numbers. They undergo characteristic degenerative changes seven days after section of the optic nerve, and this phenomenon provided the first *direct* evidence that crossed and uncrossed retinal fibres end in different cell-laminae. Each main optic fibre commonly terminates in a spray of 5 to 6 branches (at least in the small-celled laminae). The terminal boutons are in contact with the cell bodies and not with the dendrites, and it was in no case possible to find more than one bouton related to one geniculate cell. It appears, indeed, that in the small-celled laminae of the geniculate body of the monkey each optic fibre is terminally related to 5-6 cells and there is apparently no overlap of the terminals of different fibres. Clearly, such an arrangement would provide for the utmost precision in the recording at the geniculate level of a retinal image. On the other hand, the profusion of synaptic contacts in the geniculate body of the cat, together with the

overlap of different optic fibres, would presumably provide an anatomical basis for a high degree of sensitivity even in low intensities of illumination.

The efferent connexions of the lateral geniculate body. In spite of many statements to the contrary, there is now reason to believe that, at least in higher mammals, the lateral geniculate body projects entirely on to the visual cortex. Thus it has been established by several observers (especially Poliak, 74) that in the monkey all the neurones of the large- and small-celled elements undergo rapid retrograde atrophy following destruction of the area striata of the same side, and this appears also to be the case in the chimpanzee (Poliak and Hayashi, 76). It appears that the geniculate cells similarly undergo total atrophy in the rat (Lashley, 52). According to Bodian (12) occasional residual neurones do persist in areas of degeneration in the geniculate body of the opossum, and this confirms previous observations of Minkowski (63) on the cat's brain. It has already been noted that O'Leary has reported the presence of short-axone cells in the cat's geniculate body. On the other hand, the evidence of retrograde atrophy seems to exclude consideration of such elements in the geniculate laminae of monkeys and apes. Reference may here be made to the contention of Balado and Franke (4) that the neurones of the large-celled laminae in the primates have no connexion with the cortex, but send their axones to the superior colliculus. However, this conception appears to be based on atrophic changes reported to have occurred in the large-celled laminae of the human geniculate body following the destruction of the colliculi by a pineal tumour, and it is doubtful whether this evidence is adequate to controvert the results of careful experiments on lower primates.

The total cell atrophy in the geniculate body which follows ablation of the ipsilateral visual cortex in primates finally demonstrates the absence of any crossed connexions from the nucleus by way of the corpus callosum to the visual cortex of the opposite hemisphere, a connexion which had been postulated by some clinicians to explain the frequent persistence of macular vision after lesions involving the area striata of one side. A similar conclusion follows from Poliak's observation (72) that lesions of the visual cortex never lead to retrograde atrophy affecting the cells of the contralateral geniculate body. This is what might be expected from a consideration of the general organization of sensory systems in the brain for it appears certain that any crossing of ascending sensory impulses which does occur always takes place at infra-thalamic levels.

The absence of any efferent connexions of the geniculate body other than with the visual cortex (such as the geniculotectal, geniculopulvinar, geniculopretectal or geniculothalamic tracts which have from time to time been described by comparative anatomists on the basis of the study of normal material) has recently also been affirmed by the experiments of Barris *et al.* (7). These authors produced small lesions in the lateral geniculate body of cats after the corresponding optic tract had undergone complete degeneration. In these cases the ensuing Marchi degeneration was found to be confined entirely to the geniculocortical connexions (the optic radiation).

The relation of the lateral geniculate body to the visual cortex. As already mentioned, the experimental evidence leads to the conclusion that in the primates all the cells of both the small- and large-celled laminae project on to the area striata of the occipital cortex. In the macaque monkey, the number of cells in the geniculate body has been computed to be of the order of 1,800,000, and it has further been estimated that approximately 1350 of these cells send their axones to each square millimetre of visual cortex (Clark, 28). Indirect evidence also indicates that the geniculate axones are distributed evenly over the visual cortex. It is interesting to compare these results with data provided by Lashley (54) for the rat. According to these data, there are approximately 34,000 cells in the geniculate body of this animal, while the striate cortex of one hemisphere has a surface area of about 20 sq. mm. Thus in the rat about 1700 geniculate cells project on to each square millimetre of striate cortex.

The axones of the geniculate cells are responsible for forming the fibre laminae characteristic of the lateral geniculate body in the primate brain. Leaving the nucleus, they turn back in the occipital lobe of the cerebral hemisphere, as the optic radiation, to reach the striate area of the cortex. Throughout their course, the fibres of the optic radiation, running in parallel order, remain segregated in bundles in accordance with the site of their origin from the lateral geniculate body (Poliak, 73). It thus appears that the topographical localization of different parts of the retina in this primary optic centre is faithfully carried on through the whole extent of the optic radiation to the visual cortex itself. It may also be noted that it is now entirely agreed that the lateral geniculate body is the exclusive source of the fibres of the optic radiations, and conversely that the lateral geniculate body projects on to no cortical area other than the visual cortex.

Besides the ascending connexions between the lateral geniculate body and the visual cortex, descending connexions have also been described. It is commonly accepted that such connexions form part of a general cortico-thalamic system of fibres whereby all the main nuclei of the thalamus receive corticofugal fibres from the particular areas of cortex on to which they project. It is further assumed that these descending fibres either exert an inhibitory or "damping-down" influence on the sensory activities of the lower centres, or else that they perhaps activate these centres and heighten their sensitivity to in-coming impulses and thus constitute a mechanism of sensory attention (Walker, 84). However, there remains some doubt whether cortico-geniculate fibres actually do exist. They have been described by Biernond (9) in the rabbit on the basis of Marchi preparations, and Mettler (62) with the same technique has affirmed their presence in the monkey. On the other hand, Poliak (73) doubts their existence in the monkey, and Barris (5) was quite unable to detect them in the cat. Clearly this is a matter which demands further investigation with a technique which is more critical than the Marchi method.

The visual cortex. Before considering in further detail the structural basis for the cortical aspects of visual functions, it is desirable to make reference to some of the main features of the visual cortex. In the higher primates, this

area is characterized by the fact that the internal granular layer (Brodmann's lamina IV) is powerfully developed. Moreover, it is split into two strata of cells (laminae IVa and IVc), separated by a fibre layer (lamina IVb). Of the two granule cell layers, the deeper (IVc) is by far the more conspicuous, and there is some difficulty from the morphological point of view in distinguishing lamina IVa from the superjacent pyramidal cell layer (lamina III). The intricate plexus of fibres which is found in lamina IVb forms in the fresh brain a white band usually visible on macroscopic inspection and termed the "stria of Gennari." Hence the visual cortex is often called the *area striata*. In the infragranular layers of the visual cortex (and also to some extent among the fibres in lamina IVb) are occasional large cells, sometimes termed the "solitary cells of Meynert."

In lower mammals, the histological structure of the visual cortex is usually sufficiently distinctive to permit of its demarcation from adjacent cortical areas. In man and other primates, the structure is highly differentiated, so that the transition from the striate area to neighbouring areas is quite abrupt. Hence it is possible to map out the surface extent of the visual cortex with the greatest precision.

Recent studies have confirmed the earlier inferences that the area striata represents the whole of the visuo-receptive cortex. For one thing, Marchi experiments have shown quite clearly that (as already mentioned) the fibres of the optic radiations are distributed to the whole of the area, and to no other area. For another, studies of the electrical activity have demonstrated (in the rabbit) a close correspondence between the optically excitable cortex and the anatomically defined area striata (O'Leary and Bishop, 68). Lastly, the extensive studies of Lashley have demonstrated that, in the rat, the visual functions of the cortex are topographically related to the equivalent cortical area.

The organization of the projection system between the geniculate body and the striate cortex has been determined experimentally by the study of the retrograde cell atrophy which follows discrete and localized cortical lesions. Poliak (74) has found that the smallest cortical lesion (about 1 sq. mm.) which leads to detectable changes in the lateral geniculate body of the monkey always results in the atrophy of a small group of cells in adjacent parts of all six layers of the nucleus. In other words it appears that the projection unit of the lateral geniculate body in respect of the cortex is a band of cells extending radially from the hilum and involving all the cell laminae. Hence, if (as seems certain) the impulses from corresponding points in the two retinae remain distinct within the limits of the geniculate body, they must be brought into the most intimate relation as soon as they are projected on to the cortex, and it may be inferred that the cortical mechanism for the fusion of two images necessary for stereoscopic vision is very localized spatially. Further the three impulses proceeding from each spot in the retina and terminating respectively in the three corresponding laminae of the geniculate body (whatever their functional significance may be) are likewise projected on to the identical spots in the visual cortex where they can undergo immediate synthesis.

The precise site of termination of the fibres of the optic radiation in respect of the various layers of the visual cortex is still in doubt. It has for many years been commonly accepted that the stria of Gennari is mainly composed of the terminal arborizations of these fibres, for atrophy of the stria had been reported in cases of long-standing blindness. Experimental observations on monkeys, however, have not supported this view. For example, the stria remains apparently unchanged in width and density in discrete areas of the visual cortex which had been isolated sufficiently long to allow complete degeneration of all in-coming exogenous fibres (Clark and Sunderland, 32). Rundles and Papez (77) have also reported that in a mangabey monkey and a baboon, in which the optic radiations had been sectioned, the stria showed no evident change in the affected part of the area striata. While, therefore, it is certain that optic fibres penetrate into the stria and enter into its composition, it is predominantly made up of fibre plexuses derived from other sources—probably intracortical connexions of an entirely local character.

The purely histological studies of Cajal had led him to infer that the optic fibres terminate in the visual cortex mainly by arborizations among the granule cells of lamina IV, though he described some extremely fine collaterals which are given off into the infragranular layers. Such collaterals have also been recorded in the cat by O'Leary (67). In the rabbit, O'Leary and Bishop (68) found that the terminal fibres appear to end mainly in the internal granular layer and perhaps also in the deeper part of the lamina pyramidalis. In the cat, they are more nearly confined in the terminal distribution to lamina IV (O'Leary, 67). In higher mammals where the layers of the visual cortex become much more sharply differentiated, it may be supposed that the laminar distribution of optic terminals is correspondingly more sharply defined, and there is some evidence that this is the case. Poliak, on the basis of Marchi experiments in the monkey, concluded that the fibres end almost entirely in lamina IVc though some also reach laminae IVa and IVb. Lastly, the study of degenerative changes affecting the visual cortex after interruption of the optic radiations led Rundles and Papez to infer that the latter terminate, to a large extent if not entirely, in lamina IVc. Poliak's observations seem to show that the optic fibres, consisting of comparatively thick axones, ascend very obliquely into the cortex from the subjacent white matter, and are thus rather distinct from the radiated bundles of fibres which are so conspicuous in the area striata and which appear to be mainly association fibres. This conclusion was strongly substantiated by the study (referred to above) of structural changes in isolated areas of visual cortex in the monkey. In a recent study by O'Leary (67) it is stated that, in the visual cortex of the cat, optic fibres may give off horizontal branches which extend for a considerable distance from the parent stem. "Such observations" (he says) "have significance in that they suggest that a generous area is the minimum size of a cortical point in the usual concept of 'point-to-point' localization." However, he emphasizes that this observation relates only to the cat, and there is reason to suppose that in the primates the distributional area of the optic terminals in the visual cortex must be ex-

tremely limited. Mention should be made of the hypothesis put forward by some authors that crossed and uncrossed retinal impulses are carried separately to each of the two layers of granule cells which comprise lamina IV in the visual cortex of primates. There is, however, no objective evidence for such an assumption.

Association fibres of the visual cortex. Consideration may now be given to the method of diffusion of visual impulses from the point where they impinge upon the cortex. It has generally been assumed that a rich system of association fibres connects the area striata with adjacent and distant cortical areas in the parietal and temporal lobes, and even extends as far forward as the motor area of the precentral gyrus, or toward the frontal pole of the hemisphere. These tracts have been supposed to be the anatomical basis for visuo-tactile and visuo-auditory associations and to provide an essential mechanism for the more elaborate integrative processes related to vision. Surrounding the visual cortex proper are zones of cortex (to which the terms *peristriate* and *parastriate* have been given) which were presumed to be "visual association areas," and it is fair to say that many neurologists held the conception that, while the striate cortex is essentially the receptive area for visual impressions, the latter can be immediately transferred, not only to all parts of the striate cortex itself by intrastriate association paths, but also to the surrounding association areas where visual associations can be formed. However, grave doubt exists in regard to the extent of these association tracts. It seems that their presence has been inferred partly on the basis of gross dissections of the white matter of the cerebral hemisphere (a method which has been shown to be both fallacious and misleading), and partly on the basis of uncritical studies of Marchi reactions following lesions of the visual cortex.

In a recent study (Clark, 27), extremely localized lesions of the visual cortex (in the monkey), carried out with the minimal damage to subcortical structures by the method of devascularization, failed to show Marchi degeneration extending in the area striata itself for more than quite short distances, a matter of 5 mm. at a maximum. In one experiment, degenerating fibres were traced to a narrow strip of peristriate cortex (area 18) immediately adjacent to the striate cortex. Hence it was inferred that an extensive intrastriate association system of fibres probably does not exist, and that association fibres proceeding outside the limits of the striate cortex probably only extend so far as the contiguous peristriate zone. These conclusions are in accordance with recent electrophysiological studies of the visual cortex in the monkey. Combining the method of local strychninization with the recording of electrocorticograms, Dusser de Barenne and McCulloch (34) found that, within the limits of the visual cortex, the strychnine spikes are entirely restricted to the area strychninized and its immediate vicinity, extending for a distance of not more than 1 or 2 mm. In later experiments, Bonin, Garol and McCulloch (14) found that, outside the area striata (area 17), disturbances induced by strychnine are propagated only as far as area 18. Further, strychninization within area 18 leads to disturbances which involve large parts of the same area, and are also

propagated to areas 17 and 19 as well as to the so-called "visuo-sensory band" of the cerebral cortex. Lastly, following strychninization of area 19, no disturbances are propagated to any part of the cortex except that immediately adjacent to the strychnine. On the other hand, strychninization of this area leads to the suppression of the electrical activity of the entire cortex. This last observation suggests the highly interesting conclusion that area 19 must project to some subcortical region which is not reached by the rest of the occipital cortex.

The immediately local effects of impulses reaching any point in the visual cortex suggest that there is no question of the activity of the area striata as a whole being a necessary condition for the completion of visual reactions at this level. This conclusion fits in well with the experimental observations of Lashley and his collaborators on the visual reactions of rats. In the first place, Lashley and Frank (55) sought for a solution of the problem whether any regions surrounding the striate cortex are essential for the retention of a visual habit based upon pattern discrimination. The results of extensive lesions involving the surrounding cortical zones led them to the conclusion either that there is no visual association area which is an essential functional element in habits based on the discrimination of simple geometrical figures, or, if there is such an area, its parts are equipotential for the habits. However, they are of the opinion that "it seems quite certain that no association area outside of field 17 is essential for the retention of the visual habit"; for, in a series of cases, all possible transcortical connexions of the striate areas were severed without abolishing the visual reactions. Subsequently, Lashley (54) found that the ability to discriminate visual figures was retained in animals in which an astonishingly large proportion of the area striata had been destroyed on both sides. In one case the lesion was so extensive that only 700 neurones were left intact in the geniculo-striate system of one side, i.e., about 1/100th of the total normal number. Lashley concludes that the simpler integrative processes involved in visual discrimination and learning may be carried out in a normal or nearly normal manner by a minute remnant of the visual cortex. He further notes that the 700 geniculate cells found effective for detail vision would cover, near the fixation point, 1/200th of the visual field, and that this would be included by a visual angle of about 14°.

It has been proved conclusively by Lashley's work that, in adult rats, destruction of the visual cortex (area 17) results in an inability to discriminate patterns. Experiments by Tsang (83) in young animals, however, suggests that this function may be taken over by the vicarious functioning of other areas. For example, he found that in one experiment in which the visual cortex had been destroyed in a 22-day old rat, pattern vision appeared to be still partly possible, and he notes that "this is inexplicable by the neurological data in hand." However, further observations of a similar kind are required to substantiate and elucidate this conclusion.

The projection of the retina on the cerebral cortex. Having determined that the whole of the lateral geniculate body projects on the visual cortex, the question

arises whether this projection conforms to any plan of localization. Here the evidence is quite unequivocal, for it has been shown that small localized lesions of the visual cortex are always followed by small localized zones of retrograde degeneration in the lateral geniculate body. Indeed, the localization is so sharp and precise that it is possible to speak practically of a point-to-point projection of the lateral geniculate body on the cortex, at least in the higher primates (including man). Even in the lowly opossum a localized visual cortical projection is present, though the precision of localization is not as great as in higher mammals (Bodian, 12). In this animal comparatively larger lesions of the cortex are required to produce distinct areas of complete atrophy in the lateral geniculate body; lesions involving less than 1/60th of the total striate area produce no demonstrable degeneration. In the rat, the localization is rather more precise (Lashley, 52).

By determining the projection of the retina on the lateral geniculate body in monkeys from the study of transneuronal degeneration following small retinal lesions, and the projection of the lateral geniculate body on the cortex by retrograde degeneration in the former following small lesions in the latter, it is obviously possible to map out the whole of the visual cortex in terms of different areas of the retina. Such charts have been completed for the monkey, and clinical observations have allowed the construction of similar charts in the human brain. Indeed, it is now possible to state with considerable accuracy the localized spot on the surface of the visual cortex which receives impulses from any localized spot in the retina.

Poliak's work (73, 74) comprises the most complete presentation in recent years of the organization of the projection system in the monkey from the retina through the lateral geniculate body to the striate cortex, and for the details of the retinal representation in the striate cortex of the monkey's brain reference may be made to his monographs. That a similar organization exists in the anthropoid apes has been shown by Poliak and Hayashi (76) for the chimpanzee, and in man by a variety of observations on clinical cases (see especially Brouwer, 16, and Balado and Franke, 4). It may be emphasized that in the area striata of each cerebral hemisphere homonymous halves of both maculae as well as homonymous halves of extramacular quadrants are represented. There is no question, therefore, of a bilateral cortical representation of the entire macula. Poliak has shown that topographical factors as well as the details of vascular supply explain why central vision is so frequently retained in pathological lesions of the occipital lobe in man.

Intrinsic organization of the visual cortex. Much requires to be done in the analysis of the neuronal pattern of the visual cortex in order to determine in what possible directions the diffusion of incoming visual impulses is likely to occur. The study of Golgi preparations of the visual cortex of primates has given some general indication of this pattern. For example, it is evident that the short-axone cells of the internal granular layer (the functionally receptive layer) can serve as interneurons through which visual impulses may be transmitted 1, to the apical dendrites of the fusiform cells in laminae V and VI;

2, to the dendrites of cells of Martinotti in lamina V and thence by their ascending axones to the zonal or plexiform layer on the surface of the cortex; 3, to the basal dendrites of the large pyramidal cells in lamina IVb (whose axones probably proceed to sub-cortical centres), or 4, to other short-axone cells by relays of which they can be diffused through the horizontally-disposed plexus of the stria of Gennari. The majority of the pyramidal cells of the cortex, including the large solitary cells of Meynert, can be activated through their apical dendrites by impulses which are diffused through the fine fibres of the plexiform or zonal layer. It is further evident that these impulses reach the zonal layer 1, by the ascending axones of the cells of Martinotti; 2, by the vertically-running fibres of the radiated bundles, which are predominantly association fibres derived from immediately neighbouring areas of the cortex, and 3, by the fine ascending collaterals of the main axones of the pyramidal cells. It is to be hoped that such inferences, based on purely histological observations, will in future studies be confirmed and extended by the application of electro-physiological methods. In a recent study of the optically excitable cortex in the rabbit's brain by O'Leary and Bishop, attention has been brought to the possibilities of such a technique, and also to its limitations. With regard to the latter, the authors point out that the axonal substrate of the cortex shows no dominant orientation throughout; in any one plane conduction of axone impulses evidently proceeds in opposite directions at the same time so that the summation of the potential which would result would be algebraic rather than simple; all axones or collaterals which pass one electrode placed in a given dimension of the cortex may not pass another situated a short distance from it; activity in a single axone and its collaterals may progress in opposite directions simultaneously. They further emphasize the complications introduced into the anatomical pattern from the standpoint of electrical recording by the profusion of short-axone cells in the visual cortex. These cells, it is considered, may provide for the formation of reverberating circuits or self-re-exciting chains, and may also act as synchronizing agents. Lastly, O'Leary and Bishop draw particular attention to the widespread origin of the axones and collaterals which contribute to the plexuses of the lamina zonalis, on the basis of which it is inferred that "the activity of the plexiform layer should reflect the status of activity in all subjacent layers of the cortex."

The efferent elements of the visual cortex. If the geniculo-striate organization can provide the anatomical basis for visual reactions independently of surrounding cortical areas, it is clear that there must exist efferent connexions through which the visual cortex is brought directly into relation with motor mechanisms of the brain-stem and spinal cord. There is some reason to believe that these connexions are mainly established by the axones of the large ganglion cells of lamina IVb and of the large solitary cells in lamina VI, for these cells undergo a partial degeneration when the subcortical connexions of the area striata are interrupted, while other cellular elements of the cortex show remarkably little change. On the other hand, it must be admitted that retrograde degeneration is not an altogether reliable index of the extent and distribution of

efferent neurones in the cortex, for the numerous collaterals given off from the proximal part of the axones are apparently capable of permitting their survival even after section of the main distal part of the axone.

Cortico-pontine connexions. Cortico-pontine connexions from the area striata have been both affirmed and denied for a number of years by various authors. In recent years the work of Sunderland (78) has contributed additional evidence for their existence in the monkey. Mettler (62), however, concluded that none are derived from the macular region of the area striata, and he was uncertain whether any come from the extramacular regions. In the cat, cortico-pontine fibres from the striate cortex have been described by Barris, Ingram and Ranson (7), confirming earlier observations made on the same animal by Poliak (72). Probably the question of these connexions will not be finally settled until some better technique can be applied to its elucidation.

Cortico-mesencephalic connexions. The cortical control of the lower visual centres in the mid-brain is an important element in the physiology of vision. It is mediated by descending fibre paths from certain discrete areas of the cortex, though the precise anatomical details of these paths are still not fully known. In a recent review of the subject, Holmes (46) has discussed the cerebral integration of ocular movements, mainly from the clinical point of view. He refers to the well-established fact that ocular movements can be induced by stimulation of an area in the frontal cortex and also of the cortex of the occipital lobe, and he makes the following statements regarding the contrast in the functional significance of these two oculomotor regions—"All evidence at our disposal indicates that the frontal oculomotor centre is concerned in those movements and reactions of the eyes which we may call voluntary. Through it we can by an effort of will look or turn our eyes in any direction and converge them on a near object." On the other hand, "the functions of the occipital centre are more numerous. Fusion, that is, the reactions necessary to unify the separate impressions from the two retinæ, and accommodation depend on it. The movement of the eyes to an object outside central vision, in so far as it is involuntary, is also excited through the occipital cortex by extrafoveal impulses. And finally the maintenance of fixation on a point, whether it is at rest or in movement, is determined by a cortical reflex mechanism in the occipital lobes."

It is presumed that the fronto-mesencephalic fibres descend in the medial part of the cerebral peduncle, and, entering thence the tegmental region of the mid-brain, terminate in connexion with the superior colliculus, or more directly with the oculomotor nucleus. The cortico-mesencephalic fibres from the occipital lobe, on the other hand, commonly form quite a well-defined fasciculus which, in Weigert sections, can be seen penetrating the base of the pulvinar, and entering the deeper part of the stratum opticum of the superior colliculus. There is some doubt whether these fibres have their origin in the visual cortex proper (as generally supposed in the past). Poliak (73), for example, believes on the basis of his experiments on monkeys that they are derived rather from the peristriate cortex, and Foerster (35) reported that in

experiments on the human brain he was unable to elicit ocular movements on stimulation of the striate area alone, while they could be obtained on stimulation of the peristriate areas (areas 18 and 19). On the other hand, in a recent paper Walker and Weaver (85) state that they were able to elicit contralateral conjugate deviation of the eyes in monkeys by stimulation of the cortex of the area striata. Moreover, they observed some evidence of localization in this motor projection system, for when electrodes were placed above the calcarine sulcus, the movements tended to be lateral and downward, while if placed below the sulcus, the movements were rather lateral and upward.

It was stated above that in normal Weigert sections the cortico-mesencephalic fibres can be seen to enter the superior colliculus. This does not preclude the possibility that some of them also terminate in deeper parts of the mid-brain. Indeed, there is evidence that this must be so, and we may refer back to the old observations of Bernheimer (8) that movements of the eyes on stimulation of the occipital cortex can still be obtained after destruction of the superior colliculus. More recent anatomical studies have provided further evidence. In the rat (Clark, 26) fibres were traced from the cortex adjacent to the area striata to the pretectal nucleus, and a similar observation was made on the cat's brain by Barris (6). The latter also showed that stimulation of the cortical area in question (which in the cat is quite restricted) gives rise to an equal constriction of the pupils, and thus may be referred to as a specific "pupillo-constrictor area" of the cortex. Lastly, according to Mettler (62), in the monkey, cortico-mesencephalic fibres arise from the striate cortex in the region of the calcarine sulcus (but not from the macular area) and also from the peristriate areas. This author also states that from the upper lip of the calcarine sulcus descending fibres extend to reach the oculomotor nucleus, the trochlear nucleus, the central gray matter of the cerebral aqueduct, and the fasciculus longitudinalis medialis.

It will be observed that not only do the cortico-mesencephalic connexions provide an anatomical basis for the cortical control of ocular movements; they may also convey impulses which can be relayed through the tectum to motor centres of the brain-stem and spinal cord by tecto-bulbar and tecto-spinal tracts.

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TISSUE CHANGES IN VITAMIN DEFICIENCIES

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The vitamins are naturally occurring organic substances which possess unique molecular structures that the organism is unable to synthesize but which are essential components of the chemical machinery of cells. Since most organisms cannot synthesize these particular molecular configurations, such substances must be supplied preformed in the food.

Pathology is the science concerned with the responses of living organisms to injurious conditions. Non-lethal factors in the course of time have led either to patterned reparative processes correlatable with normal growth sequences or to gradual adaptations which have led to species differentiation. The more ancient the conflict between organisms and environment, the more definitely patterned are the responses, notably those following physical injury.

Deficiency of a single vitamin in the nutrition of an organism creates a condition highly improbable of occurrence in nature. Therefore, the study of the consequences of vitamin deficiencies affords a new and novel approach in pathological exploration.

The morphological changes of the vitamin deficiencies reflect the injury caused by defective chemistry of cells. In a given deficiency, this kind of injury may be peculiar to one type of cell or more generally distributed in several tissues dependent upon the particular biochemical process affected and the importance of the process to the cell type. Where possible we should distinguish between primary effects (the immediate consequences of a deficiency upon tissues in which the vitamin is operative) and secondary effects—the effect upon the organism as a whole in consequence of the loss of function of the tissues primarily affected and in general manifested by inanition. Other characteristics of the vitamin deficiencies are: *a*, that great variety in the nature of tissue injury is obtainable because of the numerous deficiencies; *b*, that differences in the degree of the deficiency may lead to a difference in response; *c*, the nature and/or degree of the response may be influenced by the state of biological activity of the organism (growth, other deficiencies, infection, etc.); *d*, that injuries of this type are experimentally reproducible—which offers great advantages for study.

The morphologic effects of a vitamin deficiency, to have scientific value, should be correlatable with known normal sequences, for which purposes the study of processes of repair following replacement therapy are usually of greatest value.

In addition to the value of this method of approach in the study of tissue responses, vitamin deficiency experimentation is a means of chemical micro-dissection of cells and affords possibilities of relating function with structure that have not yet been seriously explored.

Naturally, our selection of material has been chiefly from reports of experimental work and we have included biochemical and physiological data whenever

deemed helpful in elucidation of morphological problems. We have avoided discussion of priority and have consciously excluded numerous papers from our list of references, either because of lack of merit or because their contents were adequately summarized in the publications we have included. It has seemed advisable to incorporate a few of our unpublished observations, particularly those regarding the changes in hypervitaminosis A and on riboflavin and pyridoxine deficiencies.

VITAMIN A. A review by Bessey and Wolbach in 1939 (25) contains an adequate and carefully selected list of references to the physiology of vitamin A and to the pathology in man and animals caused by its deficiency. Other references are to be found in a non-critical review by Robertson (252).

We shall limit ourselves to brief considerations of the consequences of vitamin A deficiency upon: *a*, epithelial structures; *b*, the incisor teeth of rodents, and *c*, the skeleton. While there are marked effects upon the hematopoietic tissues, we have no proof that they are different from the inanition effects of several other vitamin deficiencies.

Epithelial tissue. The changes produced in many epithelial structures are to be regarded as the most characteristic consequence of vitamin A deficiency because they appear regardless of age and presumably in all vertebrates, though demonstrated as yet only in man, monkeys, cattle, swine, dogs, rabbits, guinea pigs, rats, mice and fowls (335). The sequence of the epithelial effect may be epitomized as follows: atrophy of the epithelium concerned, reparative proliferation of basal cells, and growth and differentiation of the new products into a stratified keratinizing epithelium. Regardless of the original function and structure of the region, this replacement epithelium is identical in all locations and comparable in all its layers with epidermis (329) (330) (115) (306) (35).

The order of the response of various organs varies somewhat with different species of animals but essentially the same organs are involved. The keratinizing metaplasia is found in: *a*, salivary glands, including the submaxillary, parotid and all accessory glands of the buccal cavity, tongue, and pharynx; *b*, the respiratory tract, including the nares, maxillary sinuses, Jacobson's organ, trachea and bronchi; *c*, genito-urinary tract, including the renal pelvis, ureters, bladder, epididymis, prostate, seminal vesicles, coagulating glands, uterus, oviducts, and accessory sex glands of the vulva; and *d*, eyes and paraocular glands, including the corneal and palpebral conjunctivae, the Harderian, intra-orbital and extra-orbital, and the Meibomian glands. In man, hyperkeratotic lesions of the skin centering about hair follicles may occur in the deficiency after puberty (109).

The mucosa of the stomach and intestines and the renal tubules do not undergo the above described changes. At most, some degree of atrophy of the intestinal glands may be attributed to the deficiency. Ulcers in the forestomach of the rat following hyperkeratosis which have been attributed to vitamin A deficiency are not peculiar to this condition (224).

In general, in vitamin A deficiency the epitheliums which atrophy and which become replaced by stratified keratinizing epithelium are those which have a secreting (chemical) function in addition to the rôle of a covering layer and whose

functioning cells are without power to divide. Repair, therefore, takes place from focally distributed basal cells which multiply, spread beneath the original epithelium, and finally, through coalescence of areas thus produced, form a continuous epidermis-like layer. On the other hand, epithelial cells with chemical rôles, as liver and renal tubules, which do have the power of dividing, do not exhibit marked degrees of atrophy nor are they replaced by keratinizing epithelium. Certain stratified epitheliums—cornea, renal pelves, ureters, and bladder—in vitamin A deficiency increase their growth rate and become hyperkeratotic, which might be interpreted as evidence that these epitheliums normally have specialized functions which are inhibited by the deficiency. No satisfactory explanation has been found for the fact that the reparative activities of basal cells of many different epitheliums in vitamin A deficiency end in an identical stratified keratinizing epithelial product. A natural assumption is that vitamin A is necessary in some chemical process uniquely related to normal differentiation and life of this type of cell.

In recovery following vitamin A administration, in spite of the common morphology, the epithelium of each region returns to its normal type in morphology and function. The recovery sequences have been followed by Wolbach and Howe (331) and some of their conclusions are: "all cells of the lowermost layer of the replacement epithelium have proliferative powers as in the stratum germinativum of epidermis"; "The important histological features of repair involve removal of the layers of cells irreversibly differentiated toward keratinization and direct differentiation of the stratum germinativum into the normal type. These take place simultaneously."; "The histological sequences observed in the removal of cells above the stratum germinativum indicate that autolysis as shown by vacuolar degeneration and heterolysis as shown by leucocyte infiltration are involved."

In repair the vacuolar degeneration separates the replacement epithelium into two strata, the upper to disappear completely, the lower to reproduce the epithelium normal for the region. The sequences are very similar to those in the rat's vagina in the di-estrus. As the metaplasia of vitamin A deficiency and its recovery is a cycle that probably does not occur in animals in natural habitats, it is of great interest to note that the recovery phenomena follow a familiar physiological pattern.

Perhaps the fact of greatest interest in vitamin A deficiency is the preservation by the cells of the stratum germinativum of the replacement epithelium, of the identity of the original epithelium throughout the period of metaplasia. Wolbach and Howe (331) found that the cells of this layer can assume the original morphology and functions without undergoing division when supplied with vitamin A and inferred that the nuclear chromatin remained unaffected by the deficiency. In the opinion of these authors, the cycle of vitamin A deficiency and recovery affords an experimental method available for the correlation of nuclear chromatin and types of cytoplasmic activities. Searching cytological studies by competent persons would probably bring to light new relations of structural detail and physiological activities.

Mellanby, who at one time (204) (205) suggested that the epithelial changes of vitamin A deficiency are secondary to lesions of afferent nerves, has very recently (207) suggested, in disregard of the actual sequence of events, that the "fundamental change is overgrowth of epithelial cells of all kinds, keratinization and metaplasia being secondary to this overgrowth."

Teeth. Vitamin A deficiency produces profound changes in the incisor teeth of rats and guinea pigs because these structures grow continuously throughout the life of the animal and because the leading rôle in the organization of the tooth at the formative end is played by the enamel organ, an epithelial organ which in vitamin A deficiency atrophies and undergoes keratinizing metaplasia. Following the enamel organ atrophy, there is atrophy and failure of polar deposition of dentin matrix (predentin) on the part of the odontoblasts—cells of mesenchymal origin. The odontoblasts on the labial side of the tooth remain normal in appearance and continue to deposit dentin matrix in apposition to the enamel organ long after complete disappearance from the other sides.

After complete enamel organ atrophy in the rat, the odontoblasts disappear also on the labial side. Before they completely lose their identity, they lose their columnar shape but continue to deposit dentin matrix, but on all sides—in centrifugal fashion, like osteoblasts. Wolbach and Howe (332) whose observation we have been quoting, characterized the odontoblast as a polarized osteoblast and regard the enamel organ as the polarizing agent. They also described, in complete vitamin A deficiency, total failure of dentin formation and inclusions of enamel epithelium brought about by plication, occasioned by stress upon imperfect dentin. The above processes bring about a marked change in the structure of the tooth, abnormally thick dentin on the labial side and abnormally thin elsewhere. In recovery following vitamin A administration, enamel organ regeneration takes place and new formation of dentin is resumed by cells, apparently derived from the pulp, before they have assumed the normal columnar shape of odontoblasts.

Boyle (41) has described, in the tooth germs of a human infant with vitamin A deficiency, changes in the enamel organ comparable to those in rodent incisor teeth.

The work of Wolbach and Howe is confirmed, with but minor reservations, in a thesis in 1938 by Pohto (240). Schour, Hoffman and Smith, in a recent publication (262) also confirm on the whole and extend the findings of Wolbach and Howe. Their paper is valuable for references, for an admirable account of the histophysiology of incisor teeth of rats, additional observations and exact nomenclature. They point out that "the reaction in vitamin A deficiency offers ideal material for the analysis of a number of physiologic processes in tooth development. Their conclusions of greatest significance agree in attributing organizing influence of the enamel organ (odontogenic epithelium) upon the incisor tooth throughout the life of the animal, and they call attention to differences in the rôles of the lingual and labial odontogenic epithelium. The dentin covered by enamel (labial side) they show, forms at an accelerated rate, while the cementum-covered dentin (lingual dentin) forms at a decelerated rate. Orten,

Burn and Smith in 1937 (222) (52) by subjecting rats to long periods of incomplete intermittent vitamin A deficiency, obtained tumor-like formations and tooth duplications at the formative end of the incisor teeth. This is to be explained by the organizing action upon mesenchymal tissues of portions of the odontogenic epithelium which became displaced or isolated as the result of mechanical effects upon the defective dentin near the formative end.

The reviewers believe that careful reading of the papers cited on teeth (332) (41) (240) (262) (52) will suggest a number of problems we have not mentioned which are amenable to analysis by means of vitamin A deficiency and repair experiments.

Bone and nervous system. Paralysis and nerve degeneration as a consequence of vitamin A deficiency have been affirmed and denied by a number of investigators (25) (337). It has been known for many years that vitamin A deficiency retards growth of bone and in particular endochondral bone formation (329) (305) (146). This effect has not been regarded as different from that of inanition, however produced. Recently it has been shown by us (336) (337) that if vitamin A deficiency is established at a sufficiently early age, skeletal growth becomes retarded in a unique manner a considerable period before the rate of increase in weight is materially affected. The central nervous system and other soft tissue continue to grow at approximately their normal rate until general inanition effects appear, as shown by stationary weight or loss of weight.

The effects of this disproportionate growth of bone and central nervous system are: *a*, overcrowding of the cranial cavity, resulting in distortion of the brain, dislocation toward the foramen magnum and multiple herniations of the cerebrum and cerebellum into the venous sinuses of the dura at sites of arachnoidal villi; *b*, overcrowding of the spinal cord and herniations of nerve roots into intervertebral foramina and into the bodies of vertebrae; *c*, mechanical damage with subsequent irregular degeneration of the nerve roots, peripheral nerves and of nerve fibers in various tracts of the spinal cord and in the brain.

The reparative powers of the neurons are not impaired—at least before the effects of general inanition become apparent—as evidenced by the prompt appearance of axon regeneration phenomena on the proximal side of the mechanically damaged nerve roots adjacent to herniations.

The nervous lesions of vitamin A deficiency thus are wholly mechanical in origin.

Weanling rats (21 days old) on a vitamin A deficient diet usually exhibit signs of nerve lesions by the 54th day of age. The later the deficiency becomes established, as shown by the effect upon growth, the less frequently does the disproportionate growth reach a degree sufficient to produce compression of the central nervous system. Vitamin A administered at 42 days of age will prevent paralysis, although dissection may show slight degrees of relative overgrowth of the nervous system. If the vitamin deficient rat is prevented from growing by an insufficient amount of food, the nervous system preserves relatively normal relations to the skeleton and no paralysis ensues. Rate of growth and not age is the important factor in the production of the disproportionate relations. Rats

maintained at a small size by insufficient amounts of a complete diet into an age period in which, in normally nurtured rats, it is not possible to produce paralysis, exhibit the disproportionate growth just as rapidly as do weanlings when placed upon a vitamin A deficient diet.

We have found a similar disproportionate growth of bone and central nervous system in young vitamin A deficient guinea pigs.

The prompt retardation of endochondral bone formation in vitamin A deficiency indicates that a specific action must be involved, but no distinctive histological appearance has been found. Osteogenesis *per se* is not inhibited because bone matrix (osteoid) formation continues late into the deficiency. We have confirmed the formation of excess periosteal bone in relation to the bony labyrinth of the ear in dogs, as reported by Mellanby (206) and have recorded (337) similar findings in rats and guinea pigs. We have not found excess bone formation in rats in other parts of the skeleton. There is no explanation for the bone formation of periosteal origin in relation to the bony labyrinth of the ear. One premise of promise is that the bony capsule of the labyrinth attains adult size before birth. However, meager information at hand (213) (214) indicates that in the calf, bone proliferation in relation to the optic foramina is the conspicuous intracranial productive response of bone to vitamin A deficiency. The solution probably will require accurate information about the order of development of centers of ossification and the progress of endochondral bone formation at the base of the skull. Also, the effect of pressure upon the cranial bones, in general, must be analyzed.

Mellanby in a recent paper (207) describes in dogs overgrowth of bones; of the cranial bones, particularly those forming the posterior fossa, the vertebrae and femurs. He regards this overgrowth as related to degenerative changes in the brain, cranial and peripheral nerves. He states that "A function of vitamin A is to influence the structure of growing bone, probably by limiting the number and degree of activity of osteoblasts and osteoclasts. In its absence from the growing dog, osteoblastic and osteoclastic activity is increased, thus resulting in proliferation of cancellous at the expense of compact bone and causing many bones to lose their normally fine moulding and outline and to become thickened and enlarged." We cannot find, in vitamin A deficient rats, premises corresponding to Mellanby's in regard to osteoblastic and osteoclastic activities. We do not understand his statement in explanation of bone overgrowth.

Investigation of the consequences of vitamin A deficiency upon growth before weaning and in late prenatal periods promises to be informative. It is possible that the rosette formations of the retina in very young rats, described by Johnson (157), may be the result of more rapid growth of the retina as compared with that of the eyeball. A short paper by Hale (132) reports anophthalmos and other arrests of development in pigs born by vitamin A deficient sows. Mason (189) has investigated the effects of vitamin A deficiency in the pregnant rat. Fetal death is produced as a result of placental degeneration in contrast to the vitamin E deficiency effect which is upon fetal tissues. No significant injury of ova or impairment of implantation was found. Mason could find no evidence

of disturbances of endocrine functions of ovary and anterior hypophysis. Another bit of evidence that the epithelial changes in vitamin A deficiency are unrelated to endocrine physiology was produced by Mason and Wolfe (190) who found that castration (males and females) was without effect upon epithelial responses.

Atrophy of the testis in vitamin A deficiency has been known for many years (335) (187) (255). In our opinion, the atrophy of the seminiferous tubules in vitamin A deficiency as in other epithelial organs spares the undifferentiated cells, and hence recovery is possible with replacement therapy.

The consequences of vitamin A deficiency upon hematopoiesis have not revealed specific features. There is, however, in comparison with the anemias associated with other deficiencies, a heavy deposition of hemosiderin in the liver and particularly in the spleen. In replacement therapy recovery, following an outburst of erythroblastic activity in bone marrow and spleen, the hemosiderin rapidly disappears from the organs, which is presumptive evidence that the iron stored in phagocytic cells is utilized (334).

HYPERVITAMINOSIS A. Conflicting opinions are expressed (92) (310) (62) regarding the consequence of the administration of excessive amounts of vitamin A. The effects first described by Collazo and associates (63) (64) (65) of inanition, exophthalmos, loss of hair, and multiple fractures of the long bones produced by excessive doses of fish oil concentrates have been confirmed by a number of workers (289) (319). No confirmatory experiments by the use of pure vitamin A have been published, and naturally other substances in the concentrates have been suspected of producing the untoward consequences. No adequate pathological study has been reported of the sequences in skin and bone. The early bibliography of this subject is given by Wahlin (315) who reviews Agduhr's many pioneer publications on the deleterious effects of excessive amounts of cod liver oil.

Strauss (289) reported degenerative changes in kidney, liver, spleen and heart muscle, and atrophy of seminiferous tubules. The bones showed retardation of osteogenesis, cessation of endochondral bone formation, but no general resorption of bone. Osteoclasts were marked only in regions of fractures. Other than this brief and wholly inadequate account, we could find no microscopic descriptions recorded for the bone lesions produced by fish oil concentrates of vitamin A.

Vedder and Rosenberg (310) believe that the fish liver oil concentrates contain an unidentified toxic substance which is responsible for the lesions attributed to vitamin A. They found that vitamin D in proper dosage partially counteracts the toxicity for rats of the jewfish liver oil they used. Ascorbic acid, they found, almost completely counteracted the toxicity of jewfish liver oil for rats.

A few unpublished experiments by us have shown that the major effects reported by Collazo et al. are produced in young rats in 7 to 11 days by the daily administration of pure vitamin A¹ in amounts of 30,000 to 40,000 international units.

¹Crystalline product obtained from the Distillation Products, Inc., Rochester, New York.

No important change has been seen by us in any of the internal organs. Heart, lungs, liver, kidneys, pancreas and gastrointestinal tracts were essentially normal. The adrenals showed loss of lipoid vacuolization and some atrophy of the glomerular zone. The spleen in some instances was enlarged and actively hematopoietic. Skin lesions of eyelids and adjacent regions and snout had a considerable resemblance to vitamin B₆ (pyridoxine) deficiency lesions. The effects upon bone were dramatic. We found that 24 hours' fixation in Zenker's fluid was sufficient to decalcify, for sectioning purposes, the vertebral column and the shafts of the long bones, indicating marked decalcification. We are not prepared to give an adequate description of the processes, which are very apparent in the bones, nor to interpret what we have seen in terms of familiar sequences. The conditions leading to fractures evidently are decalcification and osteoporosis, accompanied by large numbers of osteoclasts. Osteoporosis was most marked in regions where remodelling of bone is a normal growth process, such as the ends of long bones and at the curvature of the tibia. The sites of fractures seemed to be determined in part by the degree of bone resorption and to the resultant of forces of muscular action.

Early effects of the hypervitaminosis in bone are: *a*, the presence of increased numbers of fusiform and osteoblastic cells in the periosteum; *b*, increased osteoclasts near the epiphyseal ends of bone and other regions where remodelling is normal to growth; *c*, small hemorrhages between bone and periosteum resembling early scorbutic lesions; *d*, apparent acceleration of growth and cytomorphosis of cartilage in endochondral bone formation; *e*, abundant osteoid in endochondral bone formation. Reparative processes in callus formation are reminiscent of those in experimental scurvy, but we have as yet no accurate knowledge of the duration of the fractures we have studied. Organization of hemorrhages into the tissues seems delayed. Osteoblasts are present in great numbers but osteoid deposition seems reduced in amount or delayed. In vertebrae and sternum, resorption of bone with accompanying osteoclasts is very striking, while endochondral bone growth processes in contrast to that in the long bones are more or less retarded.

Examination of the teeth has shown little of histological importance. Very striking bone resorption without typical reparative process was found in the adjacent supporting bone in regions most subject to stress.

Study of a larger series of animals with particular reference to histological sequences and to reparative sequences after withdrawal of vitamin A excess may require modification of some of the above statements; nevertheless, the destructive action upon bone of vitamin A as represented by the above dosages is a matter of fact. The histological evidence points to the organic matrix as the seat of the important or perhaps the initial disturbances which culminate in fractures. A hypothesis we hold at the present time is that the early consequence of excessive vitamin A administration is the acceleration of some processes of bone growth, notably: *a*, periosteal proliferation; *b*, epiphyseal cartilage sequences preliminary to endochondral bone formation, and *c*, remodelling of bone attended by osteoclasts. The vitamin A intake in these experiments was far in excess of that likely to be administered to humans.

Cornil et al. (66) in the guinea pig describe as visceral effects of hypervitaminosis A, focal necroses of the liver, enlargement of the islands of Langerhans, congestion of the spleen with ochre colored intracellular and extracellular pigmentation (hemosiderin?), hyperactive spermatogenesis and hyperkeratinization and desquamation of the skin. In the pituitary (67) in severe hypervitaminosis A, they describe loss of acidophilic cells and in vitamin A deficiency, the presence of pseudo-adenomatous accumulations of acidophilic cells.

It is not possible to appraise these two short papers on the basis of our own work with rats.

VITAMIN C DEFICIENCY—Ascorbic acid. The morphologic consequences of experimental ascorbic acid deficiency are practically restricted to supporting tissues of mesenchymal origin and may be expressed by the statement that there is failure of formation and maintenance of intercellular materials (328) (333). This definite and simple characterization explains the pathology of experimental ascorbic acid deficiency in susceptible animals and in the naturally-occurring deficiency in man (scurvy). We therefore do not think it important to describe in detail the lesions of human ascorbic acid deficiency (scurvy) or much of the early experimental work on guinea pigs. Hemorrhages, loosening of teeth, failure of wounds to heal and, in infants, extensive subperiosteal hemorrhages and separation of the epiphyses of long bones are manifestations of scurvy and all are explainable on the above simple pathologic principle.

For the pathology in man, consult Aschoff and Koch's monograph (13), the monograph of Park and his associates (232) and for human tooth pathology, Westin's monograph (320). These monographs and the book by Hess (145) give access to the important literature. An early important monograph dealing with experimental scurvy in guinea pigs is that of Höjer in 1924 (149).

The guinea pig has been used almost exclusively for ascorbic acid deficiency experimentation. In young guinea pigs on a diet completely deficient in ascorbic acid, microscopic evidence of retarded deposition of intercellular matrices, bone, connective tissue and dentin can be found as early as the seventh or eighth day (328) (46) (73). Finally, all deposition of intercellular material in growing structures ceases and there is slow resorption of the matrices previously laid down (328) (74) (111).

That the outstanding effect in the production of the pathology of ascorbic acid deficiency is failure in the formation of intercellular materials is generally accepted. Recently it has been verified in the healing of wounds in experimental human ascorbic acid deficiency (71) (153).

Wolbach and Howe (328) (333) and Hunt (153) believe that ascorbic acid is not necessary for long survival and multiplication of the cells concerned with matrix formation. The deficiency is specifically concerned with the elaboration of intercellular substance, which they regard as the product of cells. They describe in some detail (328) the change in morphology of osteoblasts that accompanies the failure in bone matrix production. In the incisor teeth of guinea pigs, the columnar odontoblasts undergo changes in size and shape which Höjer (149) and Fish and Harris (106) regard as degenerative but which in our opinion represent a change in morphology accompanying loss of a function. In

each instance, the bone and dentin forming cells take on the appearances of undifferentiated connective tissue cells.

All intercellular substances of the supporting tissues, bone, cartilage, fibrous connective tissue and dentin, have a common substructure of collagen. By collagen we wish to indicate a number of compounds of similar though not necessarily identical composition which react similarly to staining techniques. It is this protein substructure which in scurvy is either not produced or is produced in defective form.

Wolbach and Howe interpreted appearances in the incisor teeth of guinea pigs and in bone (gerüstmark) as indicative of the presence of a liquid. The prompt appearance of dentin and bone matrices in considerable volume following ascorbic acid administration, led them to advance the theory that the failure of cells to produce an intercellular matrix in scorbutus is the result of the absence of an agent common to all supporting tissues which is responsible for the setting, fibrillation, or jelling of a product which would otherwise remain liquid.

Some of their observations in regard to tooth changes were faulty, as has been pointed out by Ham and Elliott (138) and MacLean, Sheppard and McHenry (181). The results of the studies of Boyle, Bessey and Howe (46) are also not in accord with the interpretations of Wolbach and Howe (328) whose errors were in part the result of artefacts, in part the result of their failure to demonstrate amorphous calcified material between the layer of odontoblasts and normal dentin because they used Zenker's fixative. The disappearance of this calcareous material gave appearance as if an empty zone existed, an appearance which, however, is indicative of the presence, at least, of an exceedingly tenuous matrix. Neither Ham and Elliott (138) nor MacLean and associates (181) employed the recovery type of experiment in their studies. The latter, without adequate reasons, asserted that the deposits of dentin in the repair experiments of Wolbach and Howe were the effects of the deficiency before therapy. (Glasunow's (111) description of new formation of dentin in guinea pigs in recovery from scorbutus is like that of Wolbach and Howe's.) The former did not study animals in complete ascorbic acid deficiency. Neither group, because of the character of their material, was qualified to pass judgement on the "jellation theory." Dalldorf (74) (92), on the basis of his own experiences, adheres to the "jellation theory." Details brought to light in the study of the formation of collagen in recovery from experimental scorbutus (333) may be interpreted as supporting the "jellation theory."

Mazoue (198) studied sequences in granulation tissue induced in the peritoneal cavities of guinea pigs in scorbutus and in recovery following ascorbic acid therapy. He regarded his observations as confirming the work of Wolbach and Howe, though he made no specific mention of the "jellation theory."

Probably the most careful study of dentin formation is that of Boyle, Bessey and Howe (46). By the use of spaced alizarin injections they followed the rate of dentin formation in the incisor teeth of guinea pigs while on diets containing restricted amounts of ascorbic acid. They demonstrated a measurable quantitative relation between the rates of formation of dentin and the amount of

ascorbic acid administered. In guinea pigs receiving 0.1 mgm. of ascorbic acid daily, as well as those on an ascorbic acid free diet, the normally uncalcified predentin was absent. This paper contains proof that the mechanism of calcification is not affected in ascorbic acid deficiency, confirming the generally accepted conviction. Elsewhere Boyle and collaborators (43) state that in vitamin C deficiency, dentin deposition stops and previously deposited pre-dentin becomes heavily calcified. Odontoblasts become morphologically indistinguishable from other pulp cells. Boyle's study of enamel formation in guinea pigs in ascorbic acid deficiency (45) contains further proof of the continuance of normal calcification. The enamel organ, unlike mesenchymal sources of calcified structures, is not primarily affected in scurvy in the guinea pig or in the tooth germ in human infantile scurvy (42). Park and associates (232) showed that in human scurvy, calcification of cartilage trabeculae at epiphyseal junctions may be more intense than normally.

The continuously growing incisor teeth of guinea pigs provide perhaps the best opportunity for the study of the effect of ascorbic acid deficiency upon formation of a matrix. The evidence that dentin-matrix (predentin) formation ceases is complete and presumably most workers would agree that as the period of the deficiency extends, dentin formation becomes more and more subnormal—quantitatively and qualitatively. Whether or not the odontoblasts degenerate and disappear or simply lose their morphology and become indistinguishable from other pulp cells must be regarded as unsettled. The fact that the subnormal dentin last deposited becomes heavily calcified is of importance concerning the chemistry of calcification. The microscopic appearance of the remains of this dentin after decalcification indicates an extremely tenuous matrix.

Less work has been devoted to the study of bone matrix (osteoid) formation in ascorbic acid deficiency. There is general agreement that osteoid deposition ceases and that osteoblasts lose their morphology and take on the appearances of fibroblasts (328) (74) (106) (181) (111). Fish and Harris (106) confirm Wolbach and Howe's explanation (328) that the gerüstmark is derived from osteoblasts in abnormal bone formation.

No satisfactory experimental study of the effects of ascorbic acid deficiency upon cartilage has been published, in spite of the opportunities offered in epiphyseal cartilage growth. Park et al. (232) present evidence that epiphyseal cartilage continues to grow in human scurvy after osteoid deposition has ceased. Unpublished studies by us and P. E. Boyle indicate that in prolonged periods upon greatly reduced ascorbic acid intake (0.3 mgm. to 0.5 mgm. daily) the epiphyseal cartilages of growing guinea pigs become defective, apparently due to loss of firmness of the matrix. Reparative sequences have not been studied in detail. The tardy and less striking responses of growing cartilage to the deficiency emphasize the well known differences between the matrix of cartilage and those of other supporting tissues.

The mechanism by which ascorbic acid promotes the formation of collagenous intercellular substances is not known. It may be involved in the chemical mechanisms (enzymes) of the cells responsible for the synthesis of this protein

Careful cytological studies of the tissues most markedly affected by ascorbic acid deficiency are much needed. Most of the studies of the distribution of ascorbic acid within cells, as revealed by silver nitrate reduction methods, have been made upon a few organs—adrenal, corpus luteum, interstitial cells of the testis, anterior pituitary and liver—and are reviewed by Bourne (40). Beyond changes in amount and distribution of ascorbic acid in cells, no cytological effect characteristic of the deficiency has been described.

The morphologic effects of ascorbic acid deficiency, like those of other vitamins, must be regarded as the result of retarded or suppressed normal activities of cells. In the growing animal, the first demonstrable effect is upon the formation of intercellular materials largely composed of "collagen." The deficiency affects quantity and quality of intercellular materials. Changes in morphology of the cells responsible for the production of intercellular materials may reasonably be regarded as reversible and as an expression of loss of a specific function. The qualitative changes of intercellular materials formed during the period of depletion and the mechanism of osteoporosis and resorption of matrices in general require further study and new techniques.

VITAMIN D DEFICIENCY. The vitamin D effect is the prevention or cure of rickets, a condition characterized by defective growth of bone, the result of retardation or suppression of normal growth sequences in epiphyseal cartilage and in calcification of bone and cartilage matrices. According to Bills (31) there are ten sterol derivatives having this effect. For clinical and experimental purposes, artificially activated ergosterol from plants and the naturally activated 7-dehydrocholesterol from fish liver oils have been used. Both are now obtainable in pure crystalline form.

The pathologic changes in rickets are the result of quantitative changes in the serum calcium and/or inorganic phosphorus content of the blood such as to retard or prevent calcification of cartilage, in a restricted region involved in growth, and of bone matrix. This decrease of calcium-ion and/or phosphate-ion below the critical precipitating level is in turn due to the very inefficient absorption of calcium from the intestinal tract in the absence of vitamin D (175). In infants, the lack of vitamin D alone is sufficient to produce rickets, while in a more resistant species such as the rat, the lack of vitamin D must be accompanied by a "relative deficiency of calcium or phosphorus or an absolute deficiency of either or both" (274). Similar accentuating conditions are probably necessary for the production of osteomalacia—the adult counterpart of rickets. The main action of vitamin D is to re-establish efficient calcium and phosphorus absorption and consequently to restore the concentrations of these ions in the blood plasma so that calcification can take place. There is no reason to believe that the cells and matrices concerned in bone growth and maintenance are defective in rickets or are directly acted upon by vitamin D (275). Bills has written an extensive critical review on the physiology of the sterols, including vitamin D (31).

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product (collagen). The hypothesis that ascorbic acid or a derivative is part of the collagen structure cannot be excluded on the basis of present knowledge. Wolbach and Howe (328) found that in the destruction of tissues occasioned by operating upon a scorbutic guinea pig, substances were liberated which caused reparative changes in teeth and bones. This has been confirmed by Bessey and Boyle and has been shown to be too large an effect to be explainable by the small amount of ascorbic acid present in the damaged tissues, largely muscle (unpublished). This observation suggests that an anti-scorbutic factor (for whose synthesis ascorbic acid is necessary) may be in reserve in certain tissues. If so, it would explain the long period in man after disappearance of ascorbic acid from the blood and tissues required to produce the lesions of scurvy (71).

Tissue culture experiments have given conflicting results. Hass and McDonald (142) found that the addition of ascorbic acid to cultures of guinea pig fibroblasts did not promote collagen formation. They state that their "experimental data were in accord with the thesis of Wolbach and others that collagen is a secretory product of fibroblasts," although—"The observations failed to provide critical proof that this thesis is correct." v. Jeney and Törö had previously reported (155) that ascorbic acid was necessary for collagen formation in cultures of fibroblasts obtained from chick embryos.

Repair of wounds in guinea pigs in severe ascorbic acid deficiency is incomplete because of limited capillary formation and absence of collagen formation. The sequences of collagen deposition can be followed in such wounds following ascorbic acid administration and the relation of collagen to reticulum or argyrophil fibrils can be studied (333) (153). Wolbach (333) found that the first collagen deposited was homogeneous, then argyrophile fibrils appeared. The early argyrophilic fibril formation has been confirmed by Hunt (153) and Glasunow (111). The former refers to the argyrophile fibrils as precollagen and says that newly-formed collagen reverts to precollagen when the guinea pig is again put upon an ascorbic acid free diet.

The lesions of ascorbic acid deficiency in growing animals are mainly the result of the failure of formation of intercellular substances and the consequent weakness of supporting tissues. In the fully grown animal, lesions are much slower to appear and are mainly the result of failure of maintenance of intercellular materials. No one has succeeded in following the microscopic sequences of this failure in non-calcified tissues. In bone, osteoporosis makes the process visible; in other tissues, breaks accompanied by hemorrhages give evidence of the deterioration. The mechanical consequences in guinea pigs of long-continued inadequate supply of ascorbic acid are strikingly exhibited by the loosening and wandering of the teeth, the result of weakness of the collagen fiber suspending apparatus and of the alveolar bone (44).

In guinea pigs which received inadequate amounts of ascorbic acid (0.3 mgm. to 0.5 mgm. daily) for long periods, striking accumulations of connective tissue cells have been found, notably at attachments of muscle to bones and fascia (338). This may be interpreted as a compensatory hyperplasia, occasioned by mechanical weakness, the result of diminished collagen production.

Glasunow (111) in 1937 published fairly extensive studies of ascorbic acid deficiency in growing and in fully grown guinea pigs. His results agree with those of earlier publications. He characterizes scorbutus as a disability of all mesenchymal tissues in which they lose the property of differentiation. His account of sequences of bone resorption, osteoporosis in scorbutus, agree with those of Wolbach (333) in that an actual disappearance of bone matrix takes place with the liberation of bone corpuscles and fibrils. Ham and Elliott's (138) explanation is that in scorbutus there is failure to replace the normal breakdown, stating for a premise: "It is well known that the structure of bone is always changing; old Haversian systems constantly break down and are replaced by new. . . ." Hunt (153) for the first time observed the effect of deprivation of ascorbic acid upon newly formed scar tissue. He states that the new collagen reverts to "an argyrophil precollagenous state, very different from the comparable intercellular material in the scar of the control animal. . . ."

There are two views we may take in regard to the maintenance of intercellular materials. One is that the normal state is one of continuous breakdown and disappearance, with concurrent deposition of new materials; the other is that the normal state is one of equilibrium maintained by metabolic processes unaccompanied by physical breakdown. It is possible that new ideas may come from further studies of the effects of ascorbic acid deficiency. It has already been suggested (333) that the activities of osteoblasts may be reversible and bring about bone resorption. No important changes in blood vessels which can be attributed to ascorbic acid deficiency have been described, nor have morphologic changes been detected in capillaries. The capillary bleeding so common in scurvy is probably the result of structural weakness, either the result of changes in the cement substance binding the endothelial cells together, or in collagen fibrils immediately adjacent to the capillaries (74). New capillary formation is prevented by severe ascorbic acid depletion (333). Islands of hematopoiesis form adjacent to abortive capillary formations in the neighborhood of spontaneous hemorrhages or in the tissue surrounding blood clots after excision of muscle, in severe ascorbic acid depletion (334). The source of the blood-forming cells awaits demonstration, but the inference has been made that they are derived from endothelial cells which accumulate in consequence of failure of capillary formation (334).

Changes of importance in epithelial organs have not been proved to be a result of ascorbic acid deficiency. Atrophy of the adrenal, the result of depletion of lipids from the cortex, is a constant finding in long-continued depletion (24). Dalldorf (74) regards the keratosis of the hair follicles described by Aschoff and Koch (13) as probably due to vitamin A deficiency. The recent observation by Crandon, Lund and Dill (71) of the early appearance of similar lesions in a human subject on an ascorbic acid free diet, supplemented with "all other known vitamins," seems to prove that the epidermis is affected, particularly as the lesions disappeared after ascorbic acid therapy. Reparative proliferation of epidermis in acute severe ascorbic acid deficiency is not demonstrably impaired (328) (153).

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tions to the pathology in humans are the publications of Pommer (241) and Schmorl (259). Erdheim (97) and Pappenheimer (223) made early important studies of experimental rickets. More recent publications have contributed relatively little to our knowledge of the tissue changes in vitamin D deficiency. On all facts of major importance there is general agreement. Most of the issues of today are those of interpretation of sequences and of details. Hess' book *Rickets, osteomalacia and tetany* (147) is a convenient source of information concerning human vitamin D deficiency conditions; that of Marek and Wellman, *Die Rachitis*, deals with spontaneous and experimental rickets in domestic animals (185). Both books have lengthy bibliographies. Goldblatt's, 1931 review of experimental rickets has a bibliography of 2723 references (116).

Experimental rickets in animals duplicates completely the spontaneous disease in man and animals. The effects can all be explained as retardation of growth sequences in epiphyseal cartilage and of calcification of bone and cartilage matrices. The pathologic picture varies with the degree and duration of the deficiency (274) and in most spontaneous cases with the intermittent nature of the deficiency.

The sequences in endochondral bone formation which are disturbed in rickets are as follows: The epiphyseal plate of cartilage is firmly supported by bone on the epiphyseal side and uniformly penetrated by blood vessels of capillary dimensions on the diaphyseal (shaft) side. Growth is accomplished by continuous proliferation of cartilage cells, arranged in columns, on the epiphyseal side and concurrent degeneration of the matured cells on the diaphyseal side. The cavities occasioned by the degeneration and disappearance of the cartilage cells at the diaphyseal end of the columns are entered by capillaries accompanied by osteoblasts which form bone matrix upon the exposed cartilage matrix. The latter is calcified for a distance of two or three cartilage cells in advance (toward the epiphysis) of the entering capillaries. Endochondral growth of bone is thus achieved by a continuously retreating gap in the continuity of tissues, maintained on the epiphyseal side by continuous renewal of cartilage cells and on the diaphyseal side repaired by vascular outgrowth from the marrow comparable to repair of any defect of tissues by the process of organization or granulation tissue formation (274). The dependence of endochondral bone formation upon proliferation, differentiation, and degeneration ending in death and disappearance of the cartilage cells illustrates and epitomizes *cytomorphosis* in its four essential stages as defined by Minot (208).

The first evidences of rickets are the failure of the cartilage cells to complete the cycle of cytomorphosis and the failure of the matrix lateral to the persistent cartilage cells to calcify. In the absence of spaces created by the disappearance of cartilage cells there is no ingrowth of capillaries. Absolute rickets in the sense of the complete cessation of the above sequences is doubtful of achievement. The cessation takes place irregularly across the face of the cartilage plate. Wherever there is degeneration of cartilage cells, vascular ingrowth takes place, but the bone matrix (osteoid) deposited in the deficiency does not calcify.

The width of the epiphyseal cartilage continues to increase for a long period

because of the continued activity of the proliferative zone and the survival of the cells on the diaphyseal side. Deposition of bone matrix (osteoid) continues to take place around capillaries of the diaphysis adjacent to the cartilage. These two processes produce a zone of non-rigid tissue of abnormal width responsible for much of the skeletal distortion characteristic of the disease. In advanced rickets the non-calcified cartilage of the diaphyseal border is often transversely stratified, evidently a mechanical effect of weight-bearing. Osteoid which has accumulated is also molded by the pressure of weight-bearing. In long-continued rickets there is disappearance of the cancellous bone of the diaphysis and marked resorption of cortical bone.

The first histological evidence of repair following corrections of the diet is the presence of cleared or degenerated cells on the diaphyseal border of the cartilage, an effect visible at the end of 24 hours and accompanied by extensive vascular penetration within 48 hours. Calcification of cartilage matrix and of osteoid first takes place adjacent to capillaries which have entered spaces left by the degenerated cartilage cells, wherever this has occurred. Subsequently, the calcification of accumulated osteoid progresses toward the diaphysis. Cartilage matrix calcifies in proximity to capillaries. Excess osteoid which has accumulated during the deficiency is removed only after calcification. The removal is accompanied by many osteoclasts.

The above outline is based upon unpublished work by one of us and includes the sequences primarily occasioned by the deficiency. Dodds and Cameron (87) are in essential agreement with this account of the order of calcification and removal of osteoid in repair of rickets. We have omitted details, of interest only to pathologists, which are probably secondary to mechanical disturbances and certain consequent reparative sequences.

The facts generally accepted by investigators of rickets are: *a*, failure of calcification of the cartilage columns in the so-called zone of provisional calcification and failure of calcification of osteoid; *b*, continued growth and consequent increase in thickness of the diaphyseal cartilage and osteoid; *c*, lack of vascular growth into cartilage; *d*, resorption of bone formed before the deficiency.

Dodds (86) in normal endochondral ossification describes capillaries invading spaces occupied by cartilage cells which have not degenerated; some, he states, appear rejuvenated. Park (233) also describes capillaries invading normal cartilage cells and assigns an aggressive rôle to capillaries in endochondral bone formation and offers an explanation for the irregular penetration of capillaries too involved to present here. Harris (139) and Ham (133) as well as most textbooks, regard the cartilage cell as dead before capillary penetration takes place.

Our opinion, based upon the earliest changes to be seen in the repair of rickets, is that capillaries do not enter spaces occupied by viable cartilage cells and that the pattern of vascular penetration is determined by the distribution of cartilage cells which have completed their life cycle. The conditions necessary for this cycle are apparently the same as for those permitting calcification of cartilage and bone matrices.

An old question which has arisen from the study of rickets is whether cartilage cells transform into bone-forming cells. In the region where cartilage affected by mechanical pressure is in contact with osteoid—the rachitic metaphysis—appearances suggest that such a transformation does take place. Pappenheimer (223) avoided a discussion of the problem. Park (233) and Dodds and Cameron (88) are convinced that cartilage cells do change into cells which produce a tissue very much like osteoid. Piek (238) and Boucomont (38) describe a large scale transformation of cartilage into osteoid tissue. The evidence presented by Dodds and Cameron is worthy of careful consideration though amenable to another interpretation. In a study of normal and rachitic costochondral junctions, by means of very thin serial sections, we have not been able to obtain evidence of cartilage cells turning into osteoblasts. Bremer (47) (48) by reconstruction methods showed that in normal endochondral bone formation no osteoblasts are present in “closed lacunae which the primary marrow has not reached.”

The initiation and repair of rickets in animals offer possibilities for the elucidation of some details of bone growth and maintenance, some of which have been pointed out in another place (334).

In osteomalacia (147) (139) (238) there is pathologic resorption of bone, presumably in response to needs of vital processes for calcium. There is abundant deposition of osteoid upon the remains of the bony trabeculae and in Haversian canals and upon the inner surface of cortical bone. Osteoblasts are very numerous and the layers of osteoid are very much thicker than in growing bone. The resorption of calcified bone and failure of newly formed osteoid to calcify are responsible for the yielding of the skeleton to normal stresses.

Teeth. The condition of rickets produces its most marked effect upon human teeth during the formative periods. Defective calcification of dentin and enamel and atrophy of odontoblasts and enamel organ resulting in hypoplastic teeth are consequences described (163). Essentially similar changes occur in the growing teeth of rats (23) (165) and guinea pigs (151).

Hyperplasia of the parathyroid glands in human and experimental rickets has long been recognized. A recent paper by De-Robertis (85) contains the bibliography of the subject. His own experiments with rats show that the hyperplasia is more marked in low calcium rickets whereas in low phosphorus rickets there is hyperplasia of the thyroid. The hyperplasia of both glands is accompanied by increased complexity of the Golgi apparatus.

HYPERVITAMINOSIS D. Irradiated ergosterol administered in excessive amounts produces untoward effects in man and animals (rats, mice, cats, rabbits, guinea pigs, dogs, monkeys and fowls). Preparations containing high percentages of toxisterol are most toxic. Irradiated ergosterol contains, in addition to the antirachitic factor, calciferol, small amounts of other pharmacologically active substances. Therefore, the possibility exists that some of the effects of feeding large doses of this preparation are due not to calciferol but to these related substances.

Crystalline calciferol (prepared from activated ergosterol) in sufficiently large doses is toxic and causes the characteristic lesions of hypervitaminosis D (31).

It is the only one of the ten forms of vitamin D (31) (32) that has been used in pure form for hypervitaminosis experimentation. The character of the lesions and to a large extent their pathogenesis is the result of the exaggerated physiological effect—hyperealeemia—of excessive dosage, hence other sterol derivatives having similar physiologic or pharmacodynamic properties probably are capable of producing the pathologic picture of hypervitaminosis D. We may regard this as proved for the vitamin D of cod liver oil (activated 7-dehydrocholesterol) from the review by Goldblatt (116) of early experimentation in this field.

For the physiologic aspects of this subject, the review of Bills (31) is recommended. The pathology up to 1931 is reviewed by Goldblatt (116).

Substantially all experimental hypervitaminosis D in animals and all of the few instances of human untoward effects have been produced by irradiated ergosterol. The lesions consist of disturbances of growth sequences and of maintenance of bone and metastatic calcareous deposition in many soft tissues. Necroses in liver, kidneys and heart muscle may occur even though calcareous deposits are slight or absent (269). Whether or not degenerative changes always precede and determine the sites of calcareous deposits has not been definitely determined.

Bone changes. In conformity with the pharmacodynamics of activated ergosterol, there is variation with dosage, dietary calcium and phosphorus and age or rate of growth of the animal. The first effects of hypervitaminosis D in growing animals is upon endochondral bone formation. Moderately excessive doses of activated ergosterol accelerate or exaggerate sequences normal to bone growth at the epiphysis. Calcification of the provisional zone of calcification of the cartilage proceeds at an accelerated rate and the bones become hypercalcified (269) (275) (51). The narrowing of the epiphyseal cartilage plate and the engorgement of the capillaries penetrating the empty cartilage cell spaces (140) (258) may well be regarded as a speeding up of the cytomorphosis of cartilage essential to endochondral bone formation (334).

Larger amounts of vitamin D retard growth, presumably because of failure of growth of cartilage. The bone trabeculae which continue to form do not calcify completely, although some calcification continues apparently at the expense of cortical bone which undergoes osteoporosis (51). The degree of these changes and rate of progress are influenced by the dietary calcium and phosphate intake, shown independently by Harris and Innes (140) and Shelling and Asher (269), because the determining factor is maintenance of the elevated serum calcium level imposed by the dosage of vitamin D administered. Phosphate retention brought about by bilateral nephrectomy, accelerates the appearance of hypercalcemia and bone resorption, both of which may appear in 48 hours after excessive doses of calciferol (202). Vitamin A deficiency, because of its effect upon the kidney (keratinizing metaplasia in pelvis and ureter) as well as subtotal nephrectomy also accelerates the deposition of calcareous deposits (221).

Ham and Lewis (137) succeeded in administering to rats fed on "stock laboratory diet" amounts of activated ergosterol which produced short bones, non-

calcified trabeculae of unusual thickness and number at the epiphyseal ends, resorption of cortical bone and non-calcified matrix deposition of periosteal and endosteal origin. The growth (epiphyseal) cartilage was narrower than normal. Because of the accumulation of non-calcified matrix at the sites named, they regarded the condition as rachitic in character, disregarding the lack of the cartilage changes characteristic of rickets (274). Changes practically identical to those of Ham and Lewis had previously been described by Harris and Innes (140) who also noted that the "resorption of bone is much lessened when the diet is rich in calcium". In advanced degrees of hypervitaminosis D, resorption of bone is the most prominent feature. The most marked degree of bone resorption occurs on calcium-free diets or on diets rich in phosphorus and deficient in calcium (140) (269). Resorption of bone is of the osteoporotic type and accompanied by osteoclasia. Shelling, Asher and Jackson (270) described the relation of the bone changes to the hypercalcemia and hyperphosphatemia induced by parathormone administration in rats. These, in regard to dosage and dietary conditions, paralleled those of hypervitaminosis D. One outstanding difference was that the regions of bone resorption produced by parathormone were replaced by fibrous tissue. In general, however, the effect on bone seems to be a consequence of hypercalcemia and influenced by the dietary intake of calcium and phosphates. The comparisons by Shelling and Asher (269) (270) of the effects upon bone of excessive parathormone and vitamin D dosages in relation to calcium and phosphorus metabolism are important contributions to the histo-physiology of bone. For details of bone lesions produced by hyperparathyroidism and a comparison with those of hypervitaminosis D, consult Jaffe's review (154).

The effects of hypervitaminosis D of pregnant rats upon the bones of the offspring have been studied by Collazo, Rubino and Varela (63) and Shelling and Asher (269). The important consequences are cessation of cartilage growth and hypercalcification of bone, comparable to the effects described in rats with abundant calcium intake upon moderate over-dosage with activated ergosterol.

Teeth. Harris and Innes (140) described excessive calcification of dentin in teeth of rats and an extraordinary overgrowth of cementum. Weinmann (317) described in rats, hyperplasia and hypercalcification of both primary and secondary cementum, which were somewhat similar to the changes in bone (alveolar).

Schour and Ham (261) found that both parathormone and vitamin D in single massive doses resulted in the formation first, of a strip of dentin which was imperfectly calcified and, second, of a strip of dentin which was normally or excessively calcified. Their results, they thought, could be explained by the theory that vitamin D acts through the parathyroid mechanism.

Wilton (325) has described changes in endochondral bone formation in a human case of hypervitaminosis D which were essentially the same as those described in animals.

Lesions and metastatic calcification in soft tissues. Calcareous deposition in many tissues is plainly the consequence of hypercalcemia and, in the circumstance of abundant calcium intake, occurs before bone resorption takes place. Activated ergosterol is more potent ("toxic") in this regard than the vitamin D of fish

liver oil (activated 7-dehydro-cholesterol) (209) (141). Kidneys and blood vessels have received most attention. There are no distinctive features of the necroses (uncalcified) which have been described in liver, kidneys, and heart muscle.

The kidney, presumably because of excessive urinary secretion of calcium (119) (288) (246) is most liable to calcareous deposits. Degeneration of the kidney tubules with calcareous deposits in the lumen, within degenerated epithelial cells and between the tubules and stroma is the most constant feature. Calcification around the glomeruli and of the renal blood vessels is common (119) (283) (59). Too little attention has been paid to the exact location and nature of the deposits. According to Spies and Glover (283) in rabbits the basement membranes of tubules and glomerular capsule become thickened and hyalinized before or concurrently with the calcification. The deposits outside of tubules usually are discrete, laminated, and lobulated. Appearances after decalcification suggest the presence of incorporated organic material (59) (338).

The blood vessels of the kidneys are liable to calcification and Spies and Glover (283) are inclined to attribute the tubular degeneration to vascular lesions. Gough, Duguid and Davies (119), Steck et al. (288), Chown et al. (59) and Vanderveer (309) regard degeneration of the tubules as the result of a toxic consequence *per se*.

Practically all experimenters with hypervitaminosis D have described calcification of the blood vessels. Spies and Glover (283) found lesions in arteries and veins of the rabbit's kidney. In the former the calcareous deposits were chiefly in the media and internal elastic lamina and accompanied by hyalinization of the media. Duguid (89) made a careful study of the rat's aorta in hypervitaminosis D, and said that the lesion consists of a muscular degeneration with calcification of the media. Apparently he regarded degeneration of smooth muscle cells as a primary effect. Calcification took place first in elastic laminae, and extended into the degenerated tissue between them, but also extended into non-degenerated tissue. Duguid believes that the degeneration of smooth muscle is a probable consequence of the disturbance of calcium metabolism but does not require calcium deposition *in situ*. He described reparative proliferation, over calcareous deposits, of the intima with plaque formation. Vanderveer (309) is convinced that degeneration precedes calcification in blood vessels and other organs. His description of the rabbit's aorta in hypervitaminosis D corresponds closely with Duguid's for rats, though he thinks, contrary to Duguid, that the proliferation of the intima which is less common in the rabbit, is not secondary to lesions of sub-intimal tissues. He noted degenerative lesions followed by calcification in kidneys, stomach (muscle), heart, liver and lungs. Ham (134) found that single large doses of irradiated ergosterol will produce massive calcification in the aorta, coronary vessels and heart muscle, as early as 48 hours after administration. Microscopic sections of tissues of similarly treated rats made after 24 hours "showed nothing that would presage such an imminent catastrophe, so that calcifications do not appear to depend on degenerative changes in the recipient tissue." He found calcium deposits on elastic fibers and in degenerated and necrotic smooth muscle. Ham and Lewis (136) reaffirm the absence of degenera-

tion preliminary to calcification in the coronary vessels of the rat's heart. They state that the media calcifies first and is followed by proliferation of cells of the intima.

In general there has been insufficient precise histological study of the pathologic sequences in hypervitaminosis D. All workers are in agreement that calcareous deposits are secondary to hypercalcemia but there is disagreement concerning a direct toxic action of vitamin D upon tissues not related to the effect on the blood calcium level. Ham and Portuondo (135) found that after single massive doses of activated ergosterol, pathologic calcifications did not appear when the serum calcium was rising, but did appear in large amount when it was falling. Cowdry and Scott (69) in monkeys, after using amounts of viosterol far in excess of therapeutic dosages but not sufficient to materially affect serum calcium and phosphorus levels, found few lesions in blood vessels and kidneys not found in the controls. Their results are inconclusive for premises in the consideration of mode of action of the vitamin on tissues. Goormaghtigh and Handovsky (118) studied various degrees of hypervitaminosis D₂ (calciferol) in the dog with particular reference to renal arterioles and in particular to the juxtaglomerular neuromyoarterial apparatus. Moderate doses caused hypertrophy of both the afibrillar (sensory leiomyoblasts) and ordinary smooth muscle cells of the latter, which they attribute to a stimulation of metabolism by calciferol. Larger doses of calciferol over longer periods caused atrophy and even necrosis of smooth muscle cells but not of the afibrillar muscle cells. Arterioles and prearterioles showed degeneration of the smooth muscle of the media. Calcification they regarded as a secondary feature in the vascular lesion of hypervitaminosis D. The effect upon the juxta-glomerular apparatus, in their opinion, is a factor in the production of elevated blood pressure in hypervitaminosis D. This paper presents the strongest evidence thus far produced that massive doses of calciferol have a specific toxic action other than the effect upon calcium and phosphorus metabolism. Chown et al. (59) in addition to calcification in internal organs noted transitory calcification of the skin of the head with baldness in young rats, but did not make microscopic examinations.

A specific toxic effect of excessive vitamin D administration upon the cells of parenchymatous organs remains an unsettled question. In spite of the affirmative evidence at hand there remains the doubt which is raised by the many similarities to be found in the effects produced by experimental hyperparathyroidism. The tendency to regard tissue changes other than calcification as the result of alterations in cell environment in the period of ascending hypercalcemia is a tempting one. Knowledge of the precise location of the initial calcium deposits in soft tissues would be of considerable value. From such evidence as can be found in the literature, it is between cells. Further exploration of the problem may yield information regarding a possible rôle of intercellular materials as a temporary repository for materials which diffuse from blood vessels in maintenance of homeostasis.

VITAMIN E. For a full account of the development of evidence leading to the establishment of vitamin E as a nutritional essential, the isolation of the active

compounds, alpha, beta and gamma tocopherols from wheat germ and other plant oils, the chemistry and synthesis of these new substances and what is known of their physiology and distribution in nature, the reader is referred to recent excellent reviews elsewhere (100) (195) (16) (201) (281) (278).

Pathological lesions preventable by pure vitamin E (tocopherols) have been demonstrated by: *a*, failure of early embryonic development in the rat (98) (308) (101) (95) (193) and mouse (192); *b*, irreparable degeneration of germinal epithelium in the rat (98) (193) (186) (103); *c*, a degeneration (nutritional dystrophy of the skeletal musculature in guinea pigs, rabbits (112) (215) (273) (178), rats (99) (220) (20) (113) (93) (102) (166), dogs (10), and ducklings (226); and *d*, a nutritional encephalomalacia in the chick (225) (76) (227). In addition, degeneration of the testes of male fowls (2), early embryonic death of chicks (1), an idiopathic degeneration of the smooth musculature of the young turkey (164), and severe spinal cord lesions in adult rats (93) have resulted from feeding vitamin E low rations, but not yet shown to be preventable by tocopherols. Evidence of the need for the vitamin in other species is indirect and controversial. It consists of reports of improvement in reproduction (human, cattle, pigs) in cases of repeated spontaneous abortion, exclusive of that due to pathologic states of the uterus (313) (276) (68) (30) and of contradictory reports of benefits from treatment with alpha tocopherol in human neuromuscular and muscular dystrophies (316) (268) (199). Because of the wide distribution in natural foods (cereals, green vegetables, etc.) the deficiency of this factor has been considered as improbable in human nutrition; however, knowledge of requirements and possibilities of conditioned deficiencies due to faulty absorption or metabolism (50) remain uncertainties. Obviously, the value of vitamin E to man is not established and is in need of well-controlled investigation.

Reproductive failure in vitamin E deficient rats has been carefully studied by Evans and Burr (98) and Uner (308). Estrus, ovulation and implantation are normal, as is the morphology of the ovary and uterus. Death of the developing embryo and its resorption occur probably because of starvation and asphyxia as a consequence of limited development and failure of function of the placenta. Retarded fetal development becomes evident soon after implantation (7th to 10th day of gestation) by delay in appearance of the ectodermal cavity followed by underdevelopment and rarefaction of all the fetal tissues, especially those of mesodermal origin. The yolk sac shows a reduction in size and number of endothelial villi and blood islands. The outgrowth and differentiation of the allantois, its extension toward the trophoblast and the development of the fetal placenta are retarded, thus resulting in a delay or failure to establish maternal-fetal circulation. The death of the fetus (12-14 days) is followed by necrosis and resorption. The maternal placenta is smaller than usual and shows some distention of vessels, but is not greatly altered and continues to grow for a few days after fetal death, before regression. The uterus returns to normal, usually by the 25th day. According to Adamstone (1) the low hatchability of eggs from vitamin E deficient hens is also due to early embryonic death. In the chick, underdevelopment of fetal tissues, hemorrhage and the formation of a mesodermal

ridge ("lethal ring") around the blastoderm which chokes off the vitelline circulation to the yolk sac, causing lack of nourishment and death, is apparently the order of events.

While in rats maternal tissues, including ovaries, escape lasting damage in spite of the loss of the developing fetus, in the male the germinal epithelium becomes completely and irreparably degenerated. Vitamin E restores fertility to a few animals only, even if administered at the time of the first morphological evidence of testicular degeneration (98) (193) (191). Detailed histopathological descriptions of the testicular degeneration, first reported by Mattill (100) have been furnished by Mason (188) (191) and Evans and Burr (98). There is early loss of motility of spermatozoa and then progressive degeneration of the entire germinal epithelium beginning with the most mature cells. Fusion of the sperm, extensive nuclear chromatolysis of spermatids and secondary spermatocytes (which tend to coalesce and form giant cells) followed by nuclear and cytoplasmic degeneration of the primary spermatocytes and spermatogonia is the sequence of events, according to Mason (188). The atrophic seminiferous tubules become lined only with Sertoli cells. The interstitial cells remain structurally and apparently functionally normal, as indicated by normal gonadal hypophyseal relations. The accessory sex glands are apparently unaffected by vitamin E deficiency. According to Evans and Burr (98) the number, morphology and motility of sperm in the ejaculate are normal in the earliest stage of the development of functional sterility; at this stage loss of fertilizing power is made evident by mating experiments.

Testicular degeneration in cocks resembling that in vitamin E deficient rats has been reported after prolonged maintenance (2-3 years) on a ration treated with ferric chloride in order to destroy vitamin E (2).²

Reparable sterility as the result of degeneration of the more mature cells only of the seminiferous epithelium or of resorption of the developing embryo, may be caused by inanition or the absence of other dietary factors—such as vitamin A, which influences growth and impairs health. Mason (191) has recently reviewed this whole subject.

Although there have been numerous reports of physiological and pathological evidence relating vitamin E to the pituitary, thyroid and other glands of internal secretion, the weight of contradictory evidence makes it clear that no constant and direct relation has been established (195) (16) (201) (191) (167). Some vitamin E deficient male rats show a change in the basophilic cells of the anterior hypophysis analogous to that found in cryptorchidism and castration, but the nature of the change and the fact that the female pituitary remains normal supports the conclusion that this mild change is secondary to the testicular degeneration. The hypoplastic changes of the thyroid leading to cretinism, reported by

² This procedure has been developed in an effort to supply vitamin E deficient rations which are adequate in other respects and satisfactory for use with birds and other animals which do not do well on the purified diets. Although this leads to a diet which will produce sterility in the rat, there remain uncertainties both regarding its freedom from vitamin E and its completeness as a ration.

Barrie (19) have not been confirmed by Evans et al. (103) and Pappenheimer (228) as a constant feature of vitamin E deficiency. Mason (191) has recently reviewed the literature on the above points, and suggests the wisdom of considering slight and inconstant changes in other glands of internal secretion as secondary to the reproductive sterility until experiments prove otherwise.

Generalized severe degeneration of the skeletal muscles without emaciation or obvious lesions in other organs in guinea pigs and rabbits, as a result of vitamin E deficient diets, was first reported in 1931 by Goettsch and Pappenheimer (112) who, however, did not regard vitamin E deficiency as the cause. Nutritional muscular dystrophy, now known to be an effect of vitamin E deficiency, has been produced in several species and studied by a number of workers. Extensive references are contained in recent reviews by Evans (104), Pappenheimer (230), Mattill (197) and Morgulis et al. (215). Protection of guinea pigs (273) and rabbits (178) with alpha tocopherol does not support the contention of Morgulis (215) that more than one factor is involved. The inclusion of cod liver oil or rancid fat in vitamin E deficient diets increases the severity of the muscle lesions in guinea pigs, rabbits and goats, apparently the result of destruction of vitamin E in the diet, gastro-intestinal contents, or tissues, although a direct toxic effect upon muscle has not yet been excluded (77) (184) (196) (179) (273).

The severe paralysis with subsequent death or spontaneous recovery of apparently well nourished suckling young rats of mothers on low E diets, first observed by Evans and Burr (99), is the result of muscle lesions identical with those in the guinea pig and rabbit (112) (220) (228) and are preventable by alpha-tocopherol administration either to the new born young or the lactating mother (20) (113). Nutritional muscular dystrophy in older rats follows a more chronic course. Paralysis, developing over a period of several months and varying in severity from mild incoördination and ataxia to almost complete incapacity has been observed by numerous investigators (93) (102) (177). Early stages of the disease, before paralysis is evident, are detectable by a decreased capacity for muscular tension, lowered muscle creatin and scattered necrotic fibers (166). Severe muscular atrophy finally becomes grossly evident. The animals may live for months in an almost helpless condition. Wheat germ oil or alpha tocopherol will prevent the disease or halt the progress (93) (177). Cures have been reported when treatment was prolonged and instituted before gross paralysis was evident (166).

A vagary in distribution should be mentioned. In guinea pigs (112) the masseter and tongue muscles escape although all other voluntary muscles are affected. In rats (228) the tongue muscle alone escapes although the masseter is not constantly affected.

The pathology (112) (228) (166) of the muscle degeneration is essentially that which in human pathology has long been known as hyaline, waxy, or Zenker's degeneration and associated with infectious diseases such as typhoid fever and the pneumonia of epidemic influenza. The sequences of the muscle fiber lesions in vitamin E deficient animals as described by Pappenhemier, including reparative regeneration of incompletely degenerated fibers, are essentially the same as those described in muscle from persons who died in consequence of epidemic influenza

with pneumonia (327). The lesion is a rapidly produced necrosis of muscle fibers characterized by conversion of a part or the whole of individual fibers into hyaline structureless necrotic material, which breaks up into globular and irregularly fragmented masses. If the sarcolemma survives regeneration occurs; otherwise the sequences are those accompanying necrosis from diverse causes. In the rat the preservation of detail in necrotic fibers seems to be more common than in human muscle and calcification occurs more often. We regard the as yet undetermined (228) disturbed physiology responsible for the lesion and its correlation with the factors concerned with Zenker's degeneration in the human to be of first interest rather than the apparently non-specific nature of the morphological sequences. Facts (228) (230) of importance for premises toward a physiological explanation are: *a*, that the similarity of the lesion to Zenker's degeneration of infectious diseases in man suggests a toxic product of deranged metabolism as a cause; *b*, that muscle fibers in proximity to the blood vessels of the fascia are more apt to escape than those more remote; *c*, that unilateral section either of the sciatic nerve or of the Achilles tendon gives unilateral protection, whereas inactivation of a limb by a cast does not, and *d*, transection of the lower spinal cord has given inconclusive results.

The myocardium and the smooth muscles of the intestine and all other organs are unaffected in rabbits and suckling rats. The brain, cord, peripheral nerves, terminal nerves and end plates were well preserved (228). Olcott (220) and Barrie (20) also found a normal nervous system in newly dystrophic rats. Einarson and Ringstead (93) have described severe cord and peripheral nerve lesions preventable by wheat germ oil in their older vitamin E deficient rats, and compare certain stages of the disease (degeneration of pyramidal tract, anterior cells and dorsal sensory tracts) with amyotrophic lateral sclerosis and progressive muscular dystrophy, two important degenerative diseases in man.

Limited observations by Wolf and Pappenheimer (339) apparently confirm the occasional presence of cord lesions in the chronic deficiency. On the basis of present pathological knowledge and the contradictory clinical reports (30) (316) (268) (199) one must conclude that no relation has been established between alpha tocopherol and the neuromuscular dystrophies of man.

Paralysis due to nutritional encephalomalacia is the consequence of vitamin E deficiency in newly hatched chicks (225) (227) (4) (339). The essential lesion is an ischemic necrosis followed, if the animal survives, by reparative organization of the dead tissue. The cerebellum is most severely affected but the cerebral hemispheres, medulla and mid-brain may suffer milder injury. The vascular origin of the disease is indicated by the physiological demonstration of impaired blood supply to the cerebellum before lesions are evident, the presence of thrombi and the histopathology of the lesion. The following descriptive summary of the lesion has been given by Pappenheimer, Goettseh and Jungherr (227) "a degenerative lesion, characterized by edema, rapid necrosis of the neural elements, and later of the astrocytes and oligodendroglia. As reaction changes, one may list the multiplication and local increase of the microglia and subsequent transformation into compound granular cells, the proliferation of endothelium with the formation of new vascular ingrowths into the degenerated areas, and finally, the

partial mesodermal organization of the softened tissue. The disease is difficult to produce in chicks more than a few weeks old but may occur in the young in varying degrees of severity from mild lesions demonstrable only by section and causing no symptoms, to those involving most of the brain, easily detected grossly and resulting in severe paralysis and death. Spontaneous unexplainable recoveries are frequent."

The turkey reacts to vitamin E deficiency by a degeneration of the smooth musculature of the gizzard, while the skeletal musculature remains apparently normal³ (164). There develops a patchy hyaline necrosis of the smooth muscle fibers, accompanied by an inflammatory reaction, followed by fibrosis and attempted muscle regeneration. Ducklings respond to the same diets by developing a severe myopathy analogous to that found in mammals (226) (29). It is interesting to note that corresponding diseases have been found in chicks, turkeys and ducklings obtained from commercial hatcheries (227). Species variation is further emphasized by the fact that adult rats protected from muscle dystrophy may nevertheless be sterile (193) while dystrophic young have been born and the testicular epithelium found normal in rabbits with severe muscle dystrophy (230) (180). Another example of species variation is the occurrence of muscular dystrophy in the mouse without testicular degeneration (231). The influence of physiological state on the manifestations is shown: *a*, by the acuteness of the muscle dystrophy of young rats (99) (113) as compared with old (102) (166); *b*, the reproductive requirements of the male compared with the female rat (193) (95), and *c*, the refractivity of older chickens and turkeys (225) (164). Adamstone reports a variety of lesions occurring in chicks raised on the iron-treated diets with liver oil supplements—lymphoblastomas (3), erythrophagocytosis (5), reticulum cell sarcomas (6). These unconfirmed effects are difficult to evaluate because of the involvement of factors other than vitamin E.

The striking variety of the pre and post-natal pathological manifestations of vitamin E deficiency indicates the need for further efforts to find a common morphological or cytological characterization. The explanation of spontaneous recoveries in young rats and chicks remains a mystery. The relation of cod liver oil and rancid fats to experimental dystrophy and vitamin E should continue to receive attention. Further studies of the pathology of nutritional muscular dystrophy relative to nerve changes and the neuromuscular diseases of man are of obvious importance. Does man require vitamin E? If so, how much, and what are the manifestations of the deficiency—muscular, reproductive, or other disturbances?

VITAMIN K. No contribution to the pathology of vitamin K deficiency has been made since Dam and Schönheyder's description in 1934 (75) of "A deficiency disease in chicks resembling scurvy." In addition to hemorrhages occasioned by slight traumata, they described erosions of the lining membrane of the gizzard. Another, at present unidentified, factor is now held to be responsible for the gizzard lesions (8) (9).

³ This is to be distinguished from the gizzard erosion occurring in chickens and other birds apparently also due to the absence of an essential nutritive factor.

We have been unable to find any description of the pathology associated with vitamin K deficiency in birds or mammals which mentions more than the occurrence of hemorrhages. It is not clear that hemorrhages occur without some degree of trauma. We wonder if diminished clotting power of the blood is the complete explanation of the bleeding because it requires the assumption that in ordinary activities, with attendant minor traumatizations, the clotting mechanism is constantly being called into action in normal individuals. If a low prothrombin level does have an effect upon the vulnerability of capillaries, it is doubtful if the microscope can reveal it.

It is now known (244) (49) that sweet clover disease of cattle, in spite of the hemorrhages (260) (253) (254) is not a consequence of vitamin K deficiency *per se*. The mechanism of the prothrombin deficiency in this disease has not been worked out.

Extensive bibliographies pertaining to vitamin K are contained in the reviews by Almquist (9) and Brinkhous (49) and in the book by Butt and Snell (53).

VITAMIN B COMPLEX. Realization of the presence in certain natural products (yeast, milk, liver, etc.) of nutritional factors essential for certain mammalian and avian species and their common absence from rations usually employed in experimentation led to the association of a group of otherwise unrelated substances under the indefinite but widely used term "Vitamin B Complex." Contemporary knowledge of the factors comprising the "B complex" and the highlights of the historical development of the subject are to be found in the recent book by Eddy and Dalldorf (92) and in chapters by Nelson (219) and Sebrell (266) in the American Medical Association monograph on vitamins. Space limits this review to a brief summary of the pathology of only the best known members of this group of substances.

THIAMIN (VITAMIN B₁) DEFICIENCY. The striking manifestations of disturbed physiology of acute thiamin deficiency are accompanied by no, or at most, a few apparently simple tissue changes. Degeneration of peripheral nerves, so generally accepted as its outstanding consequence, has been shown experimentally to be a result of a more chronic course of this deficiency. Another feature of spontaneous thiamin deficiency in man (beri-beri) which also has been duplicated in animals is cardio-vascular failure accompanied by right-sided enlargement of the heart, venous congestion of the abdominal viscera, effusions into serous cavities, and edema of the extremities. Paraventricular degeneration and hemorrhage (Wernicke's disease in man) has also been reproduced in thiamin deficient pigeons and rats.

Access to the literature, historical and experimental, may be had through the bibliographies of a few comprehensive publications; the books of Williams and Spies (324), Eddy and Dalldorf (92) and papers by Cowgill (70) and Vedder (311). A recent discussion of the cardiovascular manifestations of thiamin deficiency in man and animals with a good bibliography is that by Weiss (318). An interesting recent human experimental study is reported by Williams, Mason, Wilder and Smith (321).

Thiamin requirements are proportional to the available carbohydrate used by

the organism (324) (70) (296). Therefore, the course of the deficiency can be influenced by either of these two variables. Likewise, unless efforts are made to maintain adequate caloric intake, starvation may become a compensating factor in preventing the development of typical symptoms and lesions, or in making the deficiency more chronic in appearance (296). Most of the pathologic physiology and pathology of thiamin deficiency is undoubtedly explainable on the basis of the rôle of thiamin in carbohydrate metabolism. The marked involvement of the nervous system is significant "when one considers that nervous tissue with respiratory quotient of unity is totally and uniquely dependent upon oxidation of carbohydrate for its function and integrity" (321).

Swank (295) and Swank and Bessey (296) emphasize the variation in symptoms and lesions of thiamin deficient pigeons according to the severity and duration of the deficiency.

Careful studies by Swank and Prados (297) on acutely deficient pigeons showed that selective mild degeneration of the peripheral portions of many axons from the vestibular nucleus was frequently but not invariably present in early opisthotonus, while the proximal portions of the axons and the cell bodies remained histologically normal. The earliest changes which could be recognized were swelling and deep staining of the neurofibrils in the portion of the axon involved. However, the functional origin of the opisthotonus seems evident from the rapid response (few hours) to replacement therapy. No other nerve lesions of significance were observed in the acute deficiency. Swank and Bessey (296) have found that rations inadequate in thiamin (chronic deficiency) in pigeons is followed not by opisthotonus but by leg weakness and paralysis, with degeneration of the peripheral nerve which begins at a point most distal to the cell body and progresses centrally. Thiamin therapy, if instituted before neuron death, results in recovery in a time commensurate with the known rate of axon regeneration. These conclusions were based on observations made at different levels of the sciatic nerves and the employment of suitable techniques for the study of axis cylinders and myelin sheaths. Adequate controls excluded the possibilities that these effects were due to general inanition or absence of other factors. According to Swank (295) myelin sheath changes follow those in the axis cylinder. Zimmerman (344) (345) holds the contrary view.

Prickett (242) and Prickett, Salmon and Schrader (243) found, in acutely thiamin-deficient rats, no more degenerated nerve fibers than in normal controls. In chronically deficient rats, marked degeneration of nerves was found and long-continued deficiency caused irreparable damage. Peripheral nerves only were studied by means of polarized light and Sudan III techniques upon frozen sections, so that it was not proved that the irreparable damage (paralysis of the rats) was the result of death of neurons.

In dogs also, Street, Zimmerman, Cowgill et al. (291) found that the signs of nervous origin (vomiting, stiffness, and unsteadiness of the hind legs) in acute thiamin deficiency disappeared within a few hours after administration of the vitamin. They regarded the early damage to the nervous system as functional. In chronically deficient dogs, recovery did not take place in a month during

which time large amounts of thiamin were given. No mention is made of the pathology of the acutely deficient dogs, but in the chronically deficient ones there was myelin degeneration of peripheral nerves and the posterior columns of the spinal cord. In two dogs there was gliosis in the posterior columns and in addition, in one dog, there were bilaterally symmetrical glial scars in the dorsal spinothalamic tracts, proof of irreparable injury. It seems clear that thiamin deficiency *per se* can lead to nerve degeneration. Other deficiencies which also may result in nerve damage are discussed elsewhere.

Bilaterally, symmetrical minute hemorrhagic lesions in pons, medulla, and cerebellum, in the brains of thiamin deficient rats and pigeons were first described by Priekett (242), later by Church (61). Zimmerman (345) and Alexander (7) from comparative studies of the lesions in the brains of pigeons and humans, have come to the conclusion that thiamin deficiency is the cause of Wernicke's disease in man. This disease is associated with chronic alcoholism and characterized by minute hemorrhages into nuclei around the ventricles, most constantly involving the nuclei of the extrinsic muscles of the eye (7). Alexander (7) explains the lesions on the basis of "angiodegeneration with varicose deformity of the vascular bed" and states that: "The resulting subacute necrosis of the parenchyma with glial proliferation and proliferation of endothelial and adventitial elements of the vascular walls, as well as the hemorrhage, are secondary." Swank and Prados (297) review the subject of cerebral lesions and on the basis of their experiments upon pigeons and rats come to the conclusion that the hemorrhages are secondary to degenerative changes in the neurons surrounding blood vessels. Proliferation of adventitial cells of blood vessels and gliosis they regard as reparative responses. They suggest that the vascular changes are the result of accumulation of acid metabolites in the adjacent tissues. The sequences they describe are vascular hyperemia, perivascular edema and, finally, hemorrhages.

A wholly satisfactory explanation of the hypertrophy of the right side of the heart and eventual left-sided failure has not been achieved. The edema of beri-beri and serous effusions in thiamin-deficient animals are in part to be explained by the disturbed physiology of heart and peripheral blood vessels. These subjects are discussed by Vedder (311) and Weiss (318). Swank and Bessey (298) have found that the characteristic manifestations of cardiac failure (beri-beri) can be regularly produced in the pigeon by chronic thiamin deficiency.

Numerous observers have reported hypertrophy of the islands of Langerhans in thiamin-deficient animals (335).

There is no lesion specific for thiamin deficiency in the sense that widespread keratinization of epitheliums is characteristic of vitamin A deficiency and the effects upon intercellular materials is characteristic for ascorbic acid deficiency.

The inability of cells to utilize carbohydrate sufficiently for the needs of normal processes may alone be responsible for the degeneration of neurons. Accumulation of acid metabolites has been a favorite theory in accounting for the lesions. We recommend Cowgill's (70) discussion of the physiology of thiamin. Possibilities for the investigation of some aspects of neuro-physiology may be found

through study of thiamin deficiency states, as suggested by the early neurological responses and the pathology.

RIBOFLAVIN DEFICIENCY. Riboflavin (lactoflavin), once called vitamin B₂ or G, is known to be a nutritional essential for growth and normal health for the rat (148), mouse (125), chick and turkey (172), pigeon (125), dog (263) (290), pig (152) (235) and man (302), and because of its widespread distribution and fundamental rôle in cell respiration is probably required by all vertebrates and by many lower forms (22) (280). General knowledge of the subject, methods of isolation, synthesis, function in cellular oxidation-reduction enzymes systems, human requirements, distribution in foods and relation to other members of the "B group" of vitamins can be found in recent reviews and textbooks (272) (200) (37) (271) (17) (21) (267) (217).

Lesions of the eyes, skin, mouth, tongue, and nervous systems and profound collapse with coma and death have been reported in more than one species as manifestations of riboflavin deficiency. The only histopathological studies of uncomplicated riboflavin deficiency are on the rat (92) and dog (263) (290) (292), and these are limited and sketchy. However, it seems reasonably certain from the similarity of gross appearances and other considerations that the primary tissue alterations are the same in most species.

The ocular signs and symptoms of riboflavin deficiency in both man and experimental animals are photophobia, contraction of the palpebral fissure, lacrimation, burning and itching of the eyes, soreness and swelling of the lids, visual fatigue, blurred vision, congestion of the conjunctiva and limbic plexus with ingrowth of capillaries into the cornea, and keratitis with eventual ulceration (302).

The experimental pathological studies in the rat by Bessey and Wolbach (26) and the subsequent clinical and experimental observations by Kruse, Sydenstricker, Sebrell and Cleckley (168) (301) and others (91) (284) (150) (158) definitely established that the corneal symptoms and lesions are characteristic of riboflavin deficiency and the earliest and most easily recognized diagnostic sign.

Our histological and slit-lamp studies of the developing corneal lesions in the rat showed that the "sprouting" of capillaries from the limbic plexus into an otherwise normal cornea was the first morphological indication of riboflavin deficiency. The first invading capillaries lie just beneath the epithelium and extend toward the center of the cornea. Later they penetrate the tunica propria. As the deficiency progresses, edema, cellular infiltration and separation of the fibers of the tunica propria, with resulting corneal cloudiness, appear. The corneal epithelium remains grossly unchanged until late in the deficiency and then undergoes degenerative changes which are regarded as secondary to conditions in the tunica propria. In advanced cases, the collagen fibers of the tunica propria become fused and hyalinized and newly formed fibroblasts appear in the zone of capillary ingrowth and infiltration. The superficial epithelium over such areas becomes markedly changed and separated from the deeper layers. Necrosis and ulceration may follow. Prompt regression of all lesions follows ribo-

flavin administration. The cornea may become clear and a diminished blood flow through the newly formed capillaries becomes evident within a few hours; and unless ulceration has occurred, the eye appears histologically normal within a week, except for the presence of collapsed capillaries, demonstrable only by injection methods. Slit-lamp observations and studies on man in 1941 indicate an analogous situation in most respects (302) (22). Similar lesions occur in riboflavin deficiency in the dog (292), mouse (27) and swine (235). The early observation by Spies, Vilter and Ashe (284) that certain ocular lesions frequently seen in pellagra improved with riboflavin therapy and the report by Park-Steen (234) that twilight blindness and other eye symptoms common in sprue, usually responded to riboflavin administration, were early evidences of the occurrence of this deficiency and these lesions in man.

Since riboflavin is a part of the respiratory apparatus (oxidation-reduction enzyme system) of the cell (17), one may speculate that this vascularization is a response to a gradually failing cellular respiration of the corneal epithelium. This is supported by the fact that vascularization is the usual response of the cornea to other kinds of chronic injury. In this case the handicap or injury is to the metabolic machinery of the cell in general. Therefore, the capillary ingrowth is diffuse and well developed before other morphological damage is evident.

Johnson and Eckhardt (156) report successes in the treatment of rosacea keratitis with riboflavin. This condition, of unknown etiology, is characterized by progressive recurrent focal injury, infiltration and necrosis of the cornea with capillary ingrowth and subsequent healing. Although distinguishable from the keratitis of uncomplicated riboflavin deficiency, it may represent a deficiency which is provoked or modified by an exciting agent or a constitutional factor. An alternative possibility would be that it represents the value of liberal riboflavin in healing processes of the cornea to injury of diverse causation. Improvement of some cases of early interstitial keratitis of syphilis (22) as a result of riboflavin therapy has been reported.

In addition to the early changes in the lids and cornea, Day and his collaborators (78) (79) (80) (81) have repeatedly produced cataracts in a majority of rats kept on riboflavin deficient diets longer than 70 days and finds mice, chickens and monkeys to respond likewise. Confirmation of these observations comes from Bourne and Pike (39) and others, but many laboratories (148) (26) for reasons still obscure, find cataracts only occasionally. Difference in colony susceptibility, degree of severity of the deficiency and the influence of the presence or absence of other factors in the ration are possible explanations. The earliest morphological changes in the lens are a proliferation of the epithelium and a breaking down of the fibers directly under the capsule. This progresses to complete dissolution of the fibers with formation of Morgagnian globules and conversion of the lens to an amorphous mass. Day (81) reports protection or arrest of the process with riboflavin.

Riboflavin deficiency has led to the development of dermatitis of varying degrees in most species studied (148) (263) (290) (152) (26) (248) (300) (114).

In rats on experimental rations, the distribution and appearance of the skin lesions led György (125) to differentiate clearly between riboflavin deficiency and that caused by a lack of another factor, pyridoxine (vitamin B₆). The dermatitis of the latter deficiency appears suddenly and is conspicuously severe on the paws and ears while the skin lesions of riboflavin deficiency are slow in development, generalized, and non-inflammatory in appearance. They are characterized by a dry greasy scaliness of the skin which tends to increase, a gradual loss of hair, most conspicuous in regions easy to scratch, and a subsequent replacement by a sparse fine curly coat of short abnormal hair. In time the skin becomes inelastic and greatly thickened. Lesions of the lips and mouth, similar to those occurring in man, are sometimes observed. Although skin lesions have been described by many investigators, careful histopathological descriptions and interpretations have not been reported.

We (335) (338) have not made sufficient study of the skin pathology to give a detailed description of the sequences in the development and repair of the deficiency lesions. We find that the initial responses are in the epidermis and its appendages. The vascular engorgement, so characteristic of pyridoxine deficiency, does not occur. The epidermis as a whole shows little change other than a moderate hyperkeratosis. In some locations there is slight hyperplasia of the epidermis, particularly of the snout and sides of the head, possibly related to scratching. Sebaceous glands, including the Meibomian glands of the eyelids become somewhat atrophic. There is an increased rate of shedding of hair which we believe to be the result of separation of the cornified anchoring cells from the epithelial sheaths. The outstanding and thus far, to us, distinctive feature of the deficiency is the effect upon regeneration of hair follicles and hair formation. In the late stage of the deficiency, regeneration of the hair follicles does not occur or is incomplete. Follicles engaged in hair formation during the establishment of the deficiency undergo atrophy and for a time continue to form imperfect hair. The atrophy is apparent in all parts of the hair follicle but is most evident in the matrix. The cuticular cells continue longest but undergo atypical cornification. Thus various degrees of retardation of hair production are found in a given area of skin: complete suppression, hair roots represented by loosely packed columns of cornified fusiform cells and hair roots consisting of medulla with imperfectly formed cortical substance. Sharply flexed or buckled hair follicles are common, presumably occasioned by the lack of support normally afforded by the forming hair shaft or root. In cross section, the hair roots are often oval or flat in outline. The microscopic appearances account satisfactorily for the gross appearances of the sparsely distributed hair. The gross impression of thickening of the skin may be accounted for by the persistence of many atrophic regenerated follicles because these may and often do extend to the depth of normal active follicles (i.e., to the muscle panniculus), and owing to their number, should affect the texture of the skin. In 48 hours after riboflavin therapy, there is marked restoration of normal appearances of the follicles and in 72 hours the epithelium of the follicle has assumed normal appearances. The matrix cells respond first.

On the dorsal surface of the tongue, the filiform papillae of the anterior portion exhibit an analogous retardation and defective formation of cornified cells. The pronounced effect of the deficiency upon hair formation as compared with the effect upon epidermal keratinizing sequences should eventually find explanation in the differences in composition of end products of the respective epitheliums.

An apparently similar skin condition frequently occurs in man, along with the ocular lesions of riboflavin deficiency (302) (264) (265) (300). This seborrheic dermatitis usually appears around the naso-labial folds but in some cases around the eyes and ears and occasionally in a generalized form. Like the eye lesions, the dermatitis responds to riboflavin therapy. This suggests that the hyperkeratosis follicularis frequently reported as occurring among the ill-nourished, especially in the Orient, might be related to riboflavin deficiency instead of A deficiency as previously concluded from rather inadequate evidence. Radhakrishna Rao (245) has recently studied the histopathology of this disease in India.

Conspicuous manifestations of riboflavin deficiency in man are reddening and desquamation of the lips and the development of fissures at the angles of the mouth, resembling perlache. The lesions begin with pallor at the angle of the mouth, followed by maceration and the formation of transverse fissures which are usually moist, with a light yellow exudate or crusted. A smooth tender magenta colored tongue, distinguishable from the scarlet atrophic tongue of nicotinic acid deficiency, contributes to the sore mouth. The human experimental studies of Schrell and Butler (265) (264) and the subsequent observations of others (302) of the response of spontaneously occurring cases to riboflavin therapy make it evident that the angular stomatitis and facial seborrheic dermatitis reported by many earlier writers as observed in pellagra, since pellagra, sprue, and among the ill-nourished groups in all parts of the world are due to riboflavin deficiency (264) (302) (169) (15). Diagnoses based on knowledge of the ocular, skin and mouth lesions indicate that this deficiency is not uncommon in the United States.

Riboflavin deficiency in the dog (338) and swine (235) and occasionally in the rat (27) leads to sudden collapse, coma and death, within a few hours and without apparent morphological cause for death. The onset is sudden and characterized by ataxia, weakness, inability to stand, and loss of deep reflexes of the limbs. The animal is fully conscious and without pain. The respiration is slow, becoming shallow and labored and finally failing. The condition is rapidly ameliorated by early parenteral riboflavin administration. The sudden collapse and rapid recovery indicate, as explanation, a failing chemical mechanism. This fits with the hypothesis that death is due to cellular asphyxia. Fields and Wise (105) suggest that an unexplained sudden human death they saw may have had a like cause. Schrell and Onstott (263) reported the presence of "yellow livers" and degenerative changes in the central nervous system of these dogs. Others (292) confirmed the nervous symptoms and lesions but did not regularly find the fatty livers. Street, Cowgill and Zimmerman (292) found myelin degeneration of the peripheral nerves and in the posterior columns of the spinal cord which

increased in degree with the duration of the deficiency. Although riboflavin administration largely prevented these lesions, there remains a doubt that they are direct consequences of this deficiency.

Phillips and Engels (236) (237) reported degeneration of the sciatic nerves and degeneration in the spinal cords of chicks on rations low in riboflavin and pantothenic acid. They claimed riboflavin prevented the peripheral lesions, while the cord lesions were associated with pantothenic acid deficiency. A rapid and severe paralysis of the legs was characteristic of the acute deficiency, while a slowly developing disability of the feet (curled toe) and legs followed a chronic deficiency. Notched beaks (a lesion at the junction of the upper beak and the flesh) is also reported as a sign of riboflavin deficiency in chicks (172) (173).

Eggs failing to hatch for lack of riboflavin show abnormalities of the embryos among which are degeneration of the Wolffian bodies, deformed down, edema, and dwarf size. The defective down formation is interesting because it corresponds to the skin defects and abnormal hair formation in the rat.

We have found (338) no changes in the rat which can be regarded as other than the results of inanition, in the osseous system, skeletal musculature, cardiovascular system, respiratory tract, gastro-intestinal tract, genito-urinary tract, salivary glands, spleen, liver, pancreas, adrenals, thyroid glands, and parathyroid glands. The lacrimal glands are normal but the Harderian glands undergo very definite atrophy and fail to form their characteristic yellow pigment (a porphyrin derivative). The spleen is non-hematopoietic and without hemosiderosis, in contrast to vitamin A deficiency. Following replacement therapy it becomes actively hematopoietic. The liver cells are demonstrably atrophic, without glycogen or stored fat. In severe deficiencies, the central cells are vacuolated and occasionally degenerated. The testes cease to form spermatozoa. The adrenals are moderately atrophic.

Riboflavin deficient rats often become heavily infested with lice (128). The cause is unknown but apparently nonspecific since this may also occur in other deficiencies (27).

Pinkerton and Bessey (239) have found a striking increase in susceptibility of the rat to the rickettsiae of murine typhus as the animal progresses in riboflavin deficiency. Pure riboflavin has been found protective. Search for a similar increased susceptibility to other intracellular parasites and for an explanation of the phenomenon has been unsuccessful.

NICOTINIC ACID (P-P FACTOR) DEFICIENCY. While recent events establish inadequate nicotinic acid as the principal dietary defect leading to clinical pellagra, they also confirm the long-standing belief that most pellagrins suffer from multiple deficiencies (94) (92) (284). This explains, in part, the great variations in symptoms and lesions occurring in this disease (282). A number of recent papers deal with the differentiation of individual symptoms and lesions based on specific treatment with the various pure vitamins (nicotinic acid, thiamin, riboflavin, etc.) and the relation of the responses to problems in diagnosis and treatment of pellagra (92) (286) (299) (341). There are no publications dealing with the pathology of uncomplicated nicotinic acid deficiency in either man or animals.

Pellagra and the corresponding disease in the dog (92) (144) (black tongue) thus far studied pathologically are, in all probability, the products of more than one deficiency. Since nicotinic acid cures certain of the ectodermal and entodermal lesions of these diseases, we may regard certain lesions of the skin and mucous membranes as the result of nicotinic acid deficiency. It is not clear at present whether the nervous lesions of pellagra and black tongue are specific late manifestations of nicotinic acid deficiency or a secondary nonspecific consequence of general disturbed tissue metabolism or an effect produced by the absence of some other essential nutritive factor (284) (282) (345) (342) (343). Myelin sheath degeneration of peripheral nerves and spinal cord tracts and complete degeneration of neurons have been described and are late manifestations. No specific feature in sequence or character of the degeneration of medullary sheath, nerve cells and axon are known. The prompt response to therapy indicates that most of the mental symptoms of pellagra have a physiological basis. The meager knowledge of the pathology of pellagra is summarized by Eddy and Dalldorf (92). Adequate descriptions of the gross appearances and distribution of the skin and mucous membrane lesions and all aspects of pellagra have been recently reviewed (286) (277) (266) (285) (18).

The lesions of the skin, mucous membranes and viscera of pellagra and black tongue have been described by Denton (83) (84). The important feature of his description of the skin lesions of pellagra (83) is that he assigns the primary rôle to changes in the corium consisting of edema and dissolution of collagen fibrils beneath the epidermis. Changes in the epidermis are regarded by him as secondary to those in the corium. Reparative proliferation of epidermis, vascular engorgement with eczema and finally cicatrization of corium and epidermal atrophy complete the cycle. Lesions of the tongue, mouth, and esophagus, though more persistent, undergo similar sequences to those of the skin. Dalldorf (92) confirms Denton's findings and interpretation. The fact that repair of the pellagrous skin lesion is accompanied by cicatrization of the corium is strong support of Denton's conclusion that the initial lesion is in the superficial layer of the corium. Should similar results be obtained in experimental nicotinic acid deficiency in animals, an important lead would be established for physiological studies of the skin. Denton found no lesions of significance in the respiratory tract and in solid viscera. The mucosa of the colon is commonly involved in somewhat characteristic but not pathognomonic fashion, the outstanding features of which are atrophy and cystic dilatation of the crypts of Lieberkühn. Dalldorf (92) reviews briefly other conditions in which similar lesions of the colon are found. In addition to skin lesions, nicotinic acid deficiency in swine (experimental and spontaneous) leads to severe lesions of the intestinal tract. Studies of such material should lead to a better understanding of the pathology of this deficiency in both man and animals.

Denton's study of black tongue (84) was made in dogs from Goldberger's laboratory. His descriptions duplicate those made on pellagra material. He concludes his report with a statement of great significance, in urgent need of confirmation: "The distinctive lesions of pellagra and those of black tongue of

dogs appear to have their origin in a failure on the part of the organisms to maintain the specialized supporting tissues of epithelium in various situations."

PYRIDOXINE (VITAMIN B₆) DEFICIENCY. The emergence of vitamin B₆ (pyridoxine) from the heat stabile "vitamin B complex" was brought about by György and his collaborators (124) (125) (33). György defined vitamin B₆ as "that part of the vitamin B complex which is responsible for the cure of a specific dermatitis developed in young rats on the vitamin-free diet supplemented with vitamin B₁ and lactoflavin" (124). Birch, György and Harris (33) pointed out that vitamin B₆ deficiency is most characteristically a disease of the extremities and that the lesions are not truly "pellagra-like." They suggested that vitamin B₆ should be called "rat acrodynia factor" (125).

Studies of the pathology of pyridoxine deficiency have been almost wholly upon the rat, which is the only animal that shows the characteristic dermatitis, although recent reports indicate that it is also an essential for other animals, such as the chick (160) (143), dog (182) (293), pigeon (54) (57) and pig (326). The evidence that pyridoxine is a human requirement is meager and inconclusive (287).

Comprehensive studies of the pathology of pyridoxine deficiency in the rat have been reported by Sullivan and Nicholls (294) and Antopol and Unna (12). These two papers provide adequate historical information and a complete bibliography of the subject.

The skin lesions of pyridoxine deficiency in the rat are characteristic in distribution and gross appearance and can be distinguished from the dermatitis of riboflavin deficiency, pantothenic acid deficiency and egg white injury (preventable by biotin) (127). The paws, snout and ears are involved in an acute erythema and edema, followed by desquamation which may finally lead to ulceration, especially on the paws, where rubbing becomes a factor. Sullivan and Nicholls (294) claim that involvement of the ears occurs only when other factors of the "B complex" are absent.

We (338) regard involvement of the ears (pinnae) a consequence of pyridoxine deficiency and have seen the same gross and microscopic appearances here as on the paws. Antopol and Unna (12) regard the ear lesions as most characteristic and intimate that the failure of Sullivan and Nicholls (294) to obtain them was due to the presence of small amounts of pyridoxine in the preparation of the "filtrate factor" they used in their diet. All observers agree that the skin of the trunk is not demonstrably affected by the deficiency.

A detailed resume of the skin pathology hardly seems warranted because the changes which have been described and which we have studied carefully from our own material are not amenable to interpretation, or even intelligent rationalization, with reference to retardation or suppression of normal cutaneous processes; a state of affairs probably the result of our deficient knowledge of the physiology of the skin.

The microscopic changes in the epidermis are marked increase of thickness, the result of increased number and size of the cells of the layers, particularly of the prickle cell layer (acanthosis), the stratum granulosum, and the keratinized

or cornified layer (hyperkeratosis). Mitotic figures are common in the basal layer. The capillaries and vessels of the precapillary size in the corium are markedly engorged and there is usually some edema and infiltration, chiefly with mononuclear cells (monocytes and lymphoid cells). In advanced degrees of the deficiency there is evidence of intercellular edema of the epidermis. The thickened keratinized layer may separate as a cast. Ulceration is a late effect and bacterial growth in the keratinized layer, and trauma (the result of scratching) probably contribute to its production.

Sullivan and Nicholls (294) found that the hair follicles and sebaceous glands in the regions of the specific dermatitis remained intact until involved by secondary infection. They regard a general partial atrophy of sebaceous glands and hair follicles late in the deficiency as a result of prolonged inanition. Antopol and Unna (12) regard atrophy of hair follicles and sebaceous glands in regions of the specific dermatitis as a late primary effect, which is in agreement with opinions held by us (338). Antopol and Unna (12) studied all organs of the rat. When choline was present in adequate amounts, the liver showed no lesions. They describe, but do not regard as specific, cellular changes in the adrenal glands, the most prominent being atrophic changes in the reticular zone, with which we are familiar and in agreement as to the non-specific nature. They also found atrophy of the testes with aspermia which is probably not specific, since we and others have seen identical changes in the testes in vitamin A deficiency—(see *vitamin A deficiency*). Retardation of endochondral bone formation, as seen by Antopol and Unna (12) and us (338) is not different from that resulting from inanition, however produced.

We have seen no lesions in our pyridoxine deficient rats which we regard as peculiar to the deficiency in the following organs: heart, eye, thyroid, parathyroid, trachea, lungs, paraocular glands, salivary glands, stomach, intestines, liver, spleen, kidneys, pancreas, adrenals, ovary, testis, bone and bone marrow, lymph nodes, skeletal muscle, nervous system.

We have failed to find in our own material or in published descriptions any histological feature peculiar to pyridoxine deficiency. The early marked engorgement of blood vessels may be a clue to the initial physiologic defect. Of greatest significance as yet unrevealed is the symmetrical distribution involving the extremities. We (338) have found that light is not an important factor and that sympathetic denervation on one side did not produce a difference in the effect of the deficiency.

An outstanding premise for the future elucidation of the pathology of pyridoxine deficiency is that the dermatitis can be cured or prevented by administration of the so-called "essential fatty acids" (34) (257). Birch (34) suggests: "that the physiological function of vitamin B₆ is connected with the utilization of the unsaturated fatty acids."

Pyridoxine deficiency in the dog leads to a microcytic hypochromic anemia (182) (293). Street, Cowgill and Zimmerman (293) report that after nearly a year on diets free of pyridoxine, their dogs showed cardiac embarrassment and degenerative changes in the peripheral nerves and spinal cord. The cardiac

failure was prevented by pyridoxine concentrates, but mild nerve changes were also observed in the control dogs. In the chick (160) (143) there is retardation of growth and symptoms suggestive of lesions of the central nervous system. Chick et al. (57) (58) report the occurrence of periodic convulsions (epileptiform fits) in swine and in rats maintained for long periods of time on pyridoxine-deficient diets. Protection with pure pyridoxine was reported. We have (338) occasionally observed similar convulsions in rats.

CHOLINE DEFICIENCY. The dietary importance and properties of choline logically include it among the substances being discussed in this paper. Low choline diets, especially when fat intake is high and protein low, lead to retardation of fat "turn over" through the liver and the development of fatty livers (28) (29). When the process is chronic, a diffuse fibrosis (cirrhosis) results; at least in the dog (55) and rat (36) (176). In addition to the development of fatty livers, acute choline deficiency in young rats leads to an early severe hemorrhagic condition of the kidneys and other tissues, resulting in a high mortality, but a surprising recovery is possible in animals which survive the few days of the acute period (120) (123). Cystine-rich diets produce or intensify the liver and kidney lesions while methionine, low-fat intake and choline have a mitigating effect (121). DuVigneaud (312) et al. have shown that methionine and choline are related in their action by both being methylating agents. Presumably the intensifying effect of cystine is due either to a competition for methyl groups or to improved growth with subsequent increased choline requirements. This whole subject has been recently reviewed by Best and Ridout (28) (29) and by Morgan (212) and Griffith (123). Griffith (121) (122), Christensen (60), Engel and Salmon (96), and György and Goldblatt (129) have described the lesions of acute choline deficiency in young rats as a hemorrhagic degeneration of the kidney, involution of the thymus, enlargement of the spleen and frequent hemorrhages into the myocardium, adrenal cortex, lymph nodes, eyes and lungs. The renal hemorrhages seem to appear first from arterioles at the periphery of the cortex beneath the capsule. In severe deficiencies glomeruli are incorporated in the hemorrhages, but it is not clear from descriptions that the glomerular arterioles become sources of hemorrhage. In acute fatal cases, extensive hemorrhages cause destruction of all elements of the renal parenchyma. Those animals which survive for several days or more show repair of the hemorrhagic necroses by organization and cicatrization. Although the mechanism underlying the hemorrhages is not yet clear, the function of acetylcholine as a neuromuscular mediator is suggestive of a neurovascular cause.

It seems probable that the lesions of cystine intoxication are analogous to those described above (90) because in the liver they are initiated by hemorrhagic necroses resembling those of eclampsia.

Jukes (161) (162) recently reported that choline is among the factors involved in the prevention of perosis, a condition characterized in chickens and turkeys by short, thick bones, notably of the tibia and tarsus, often with deformity and dislocation of the hock joint and slipping of the tendo-calcaneus (slipped tendon disease).

Rich and Hamilton (247) have reported the development of cirrhosis of the

liver of a type resembling Laennec's cirrhosis in man by maintaining rabbits on certain purified diets deficient in choline. Yeast prevented the lesion but the known vitamins were ineffective. Microscopically, the cirrhosis was diffuse and it was strikingly difficult to find necrotic liver cells in spite of the obvious reduction in parenchyma and the proliferation of connective tissue. The scarring appeared to be due not to repair of foci of necrosis involving many cells but rather a reaction to a more diffuse type of injury. They did not describe early stages of the liver lesions.

PANTOTHENIC ACID. Pantothenic acid was described, identified and finally synthesized by Williams and his various collaborators as an outcome of their interest in growth factors for yeast (323). It subsequently proved to be the much sought (filtrate factor) component of yeast and liver previously known to be necessary for growth and well being of the rat and for prevention of a dermatitis in chicks (170) (171) (251) (159) (307). It is an established nutritional essential for the dog (203) and mouse (256) and inconclusive evidence indicates many other species as well (210) (211). Pantothenic acid deficiency has not been reported in man perhaps because of its wide distribution in nature or a lack of knowledge of the signs and symptoms. The most significant lesion, preventable by pantothenic acid, that has been described, is a fatal hemorrhagic cortical necrosis of the adrenal glands in the rat (72) (210). According to Nelson (218) and Ashburn (14), congestion, hemorrhage, atrophy, focal and diffuse necrosis, cortical fat depletion and fibrosis occur as combined or independent lesions. The innermost zone (reticularis) is most severely affected, the damage becomes milder toward the periphery and the medulla is apparently not involved. This picture is reminiscent of the effects of certain toxins on the adrenal cortex. The sequence of events and interpretations of the lesions remain unsolved. The adrenal is reportedly not affected under like conditions in the mouse (171) (174). Other manifestations in this deficiency are alopecia, scaliness and thickening of the skin with eventual ulcerations, which occur about the nose and head and over the shoulders, flanks and abdomen of both rats and mice (171) (174) (248). Microscopically, the process appears to be a non-specific, hyperkeratotic, atrophic, desquamative dermatitis: a similar condition develops conspicuously on the feet of the chick (250). Inflammation and encrustation of the nose, mouth and eyes with closure of the latter has been observed inconstantly in the rat and chick. Paralysis with degenerative changes in the sciatic nerve and cord has been reported by Lippincott and Morris (174) in the mouse and by Philips and Engel (237) in the chick.

Absence of spermatogenesis, narrowing of the epiphyseal cartilage and lack of bone growth are common reactions to several types of malnutrition. There is some controversy as to whether pantothenic acid is involved directly, indirectly, or not at all in the greying of the hair (achromotrichia) which has been observed in a variety of species when kept on experimental rations (210) (211) (110) (11) (131). It seems reasonably clear that more than one dietary defect or circumstance can lead to greying, among which may be included pantothenic acid deficiency. Apparently the process of melanin formation in the pigment cells of the skin and hair can be influenced by a variety of factors. Many of the

lesions described above as manifestations of pantothenic deficiency are common to general tissue disturbances; perhaps this is an added indication of the importance of this substance to all cells.

OTHER FACTORS. György, Goldblatt and Miller (126) find, occurring in some of their experiments on rats, an aplastic bone marrow with anemia, leukopenia and thrombocytopenia (panmyelophthisis). A similar condition has been described by Day et al. (82) in monkeys maintained on experimental diets. Protection is secured by yeast and the term vitamin M has been suggested until the responsible factor becomes known. Severe necrotic lesions of the gum and mucosa and peridental tissues have been a feature in these deficient monkeys. Similar lesions have been described by others (304).

Pathological knowledge relative to several dietary factors, still not clearly differentiated, is so nebulous that discussion at this time seems unwise.

SUMMARY

The morphological manifestations of vitamin deficiencies may be grouped into four categories: 1, diffuse consequences expressive of inanition; 2, effects common to several deficiencies, especially degenerations of the nervous system and, with qualifications regarding distribution and fine details, lesions of the skin. Unknown factors, possibly absent from the experimental diets usually employed, may be responsible for some of the effects common to two or more of the deficiencies; 3, degenerative changes characteristic in kind and distribution, best illustrated by the cerebral lesions of B₁ deficiency and the degeneration of skeletal muscles and embryonal tissues in vitamin E deficiencies. The skin lesions of nicotinic acid, pyridoxine, pantothenic acid and riboflavin deficiencies and the ocular manifestations of the last may also be placed in this category. 4, Initial specific effects exhibited by striking changes in structural patterns, outstanding in relation to vitamins A, C and D.

In category 1 we include those effects which are similar to those of starvation as produced by inadequate amounts of a complete diet and we can list, with considerable assurance: *a*, retardation of growth including cessation of endochondral growth of bone; *b*, anemia as exhibited by decreased activity of the hematopoietic tissues; *c*, diffuse atrophy of skeletal muscle and various glandular organs, and *d*, retarded growth of hair.

In category 2 the effect of greatest importance is the degeneration of peripheral nerves and spinal cord tracts in the following deficiencies—thiamin, pyridoxine and riboflavin, and probably pantothenic acid, and possibly nicotinic acid. The nerve lesions reported as the result of vitamin E deficiency are probably secondary to degeneration of skeletal muscles. Mechanical factors, the result of a relative overgrowth of the central nervous system, cause the paralysis of vitamin A deficiency. With the exception of thiamin deficiency, the sequences of nerve degeneration have not been studied adequately. In this deficiency, myelin sheath degeneration follows degeneration of the axis cylinder as it progresses from the periphery toward the center.

The possibility of a deficiency directly affecting the formation and/or main-

tenance of myelin should be energetically explored because of its importance to the physiology and pathology of neurons.

In category 3 the outstanding degenerations which are characteristic in kind and distribution are those of vitamin E deficiency. The changes leading to early death of the fetus have at present descriptive value only because of our ignorance of the normal processes affected. The aspermia of vitamin E deficiency is permanent because the whole of the seminiferous epithelium degenerates, whereas in other deficiencies and in starvation inanition, the undifferentiated cells of the seminiferous epithelium survive. The muscle degeneration is the one consequence of the vitamin E deficiency which seems directly amenable to biochemical elucidation because the type of inactivity of the muscle secured by nerve section or by tendon cutting prevents its occurrence; this indicates that vitamin E may be directly concerned in the more or less known metabolic processes involved in muscle kinetics. If carbohydrate metabolism is at fault in the production of the muscle degeneration, reactions must be concerned unlike those in which thiamin operates because deficiency of the latter is not attended by skeletal muscle degeneration.

The nervous system lesions of thiamin deficiency presumably have origin in arrested carbohydrate metabolism; their localization may be an indication of relative metabolic rates of the neurons affected.

The hemorrhages of choline deficiency and vitamin K deficiency have yet to be elucidated, but indications point to a neurovascular mechanism for the former and to an unknown physiological rôle of prothrombin for the latter.

Category 4: vitamins A, C, and D deficiencies belong here. Each is characterized by important changes in structural patterns. The morphological sequences of each are easily followed and the reparative responses following replacement therapy are highly informative.

In vitamin A deficiency two apparently unrelated effects are outstanding; one the atrophy of many epitheliums with replacement by squamous keratinizing epithelium which occurs regardless of age; the other—of importance only during the period of rapid growth—a degree of retardation of skeletal growth in relation to the growth of the central nervous system, which results in mechanical compression of brain, spinal cord, and nerve roots. The epithelial effect may be manipulated, by means of periods of replacement therapy, for the study of fundamental problems in cytology, particularly in relation to such shifts in physiology as the morphological changes we have described indicate. The continuously growing incisor teeth of rodents provide opportunity for further studies upon the differentiation of cells through subjecting the mesenchymal tissue to intermittent organizing influences from the enamel organ as it responds to periods of vitamin A deficiency and recovery.

Understanding of the mechanism of the bone destruction caused by greatly excessive amounts of vitamin A should be achieved by means of coordinated biochemical and pathological studies, such as we now have in progress. The solution should give premises of value for the consideration of various aspects of bone physiology and pathology, and possibly a clue to the biochemical rôle of vitamin A.

The structural changes in ascorbic acid deficiency are the result of the failure of formation and maintenance of intercellular materials. Specifically this narrows down to problems of tissue collagen formation and its maintenance. We believe that our present knowledge of intercellular physiology can be extended considerably by careful studies of the occurrences which accompany intermittently induced vitamin C deficiency, as exhibited in regions of normal growth or in experimentally induced repair processes. Experimental vitamin deficiency pathology as well as human pathology and physiology provide many premises for the belief that collagen formation may be a reversible process. Vitamin C deficiency experimentation affords the best means of attacking this and other problems of supporting tissues suggested by their ever-changing pattern accompanying growth.

Vitamin D deficiency structural changes are explainable by the simultaneous occurrences of two conditions, one the retardation or suppression of normal growth sequences in epiphyseal cartilage, the other failure in calcification of bone and cartilage matrices. Apparently the failure of mature cartilage cells to degenerate and failure of matrices to calcify are results of a common factor—deficiency in calcium and/or phosphorus ions in the blood and extra-cellular materials. Nevertheless, the chemistries involved may be very different; an enzyme system in cartilage cells and critical precipitating levels in matrices.

The phenomena of hypervitaminosis D indicate that vitamin D in addition to increasing absorption of calcium and phosphorus from the intestines, is responsible for establishing a system in the blood plasma which withdraws calcium and phosphorus from available sources, including the bones. The demand for these elements and the ability to hold them in solution seem to have quantitative relations with the amount of vitamin D introduced. The deposition of calcium salts in soft tissues as the blood calcium level falls following a brief period of hypervitaminosis D has not been carefully studied in regard to the exact locations of deposition and relations to intercellular materials.

A few considerations should be kept in mind in planning vitamin deficiency experiments either for the purpose of characterization of a deficiency or for the elucidation of normal processes of growth and function. One is that the dietary regimen should be optimal in every respect excepting the content of the vitamin concerned, because other conditions which retard the general metabolic rate may decrease that vitamin requirement. A second is that the consequences of a complete deficiency may be so severe as to prevent survival of the animal for a long enough period for the development of distinctive functional and structural changes, at least in demonstrable forms. Acute thiamin deficiency affords an example.

A third is that the more rapid the growth rate the greater is the effect upon processes concerned in synthesis of structural materials. Ascorbic acid deficiency and vitamin A deficiency are examples.

A fourth is that there are important species variations, ranging from complete independence of a vitamin, as is the case of most vertebrates for ascorbic acid, to minor differences such as the failure of most laboratory animals to develop

the characteristic skin lesions of pyridoxine deficiency exhibited by the rat; the persistence of spermatogenesis in the mouse in vitamin E deficiency, the independence of the rat for vitamin D when provided adequate Ca and P in the food and presumably variations in skeletal effects of vitamin A deficiency as suggested by the few studies on the rat, dog and cow.

The usefulness of morphological studies of vitamin deficiency conditions extends in several directions. There are obvious applications to problems in general biology. For the physiologist and biochemist, the great value is the information to be obtained suggestive of the kind of metabolisms in which a vitamin is involved. We know, by way of problems amenable to biochemical attack, that ascorbic acid is necessary for collagen formation; that vitamin A is necessary for the functions of various epitheliums though not for their growth and survival and also for bone growth in an as yet undetermined specific manner and for vision; that vitamin E is necessary for skeletal muscle metabolism and for survival of early embryonic tissues indicative of its participation in fundamental vital processes.

The correlation of chemical studies of blood, tissues and excreta with the structural changes in bone produced by avitaminoses A, C and D and hypervitaminoses A and D and by parathormone experimentation offers promise of achieving understanding of many problems of bone physiology and pathology.

There is great need, in neuropathology, for information about myelin, its formation, its maintenance, and its physiological rôle. Extended studies of the several vitamin deficiencies apparently concerned in the causation of myelin degeneration are indicated.

The various disorders of the skin and its appendages caused by vitamin deficiencies, notably those of the B complex, suggest possibilities of obtaining fragments of information about skin physiology. Present knowledge is suggestive that in the same animal there are local differences in skin physiology.

Another field of promise is the careful study of the bone marrow for specific changes accompanying deficient formation of blood cells.

We conclude with the expression of opinions that much desirable information is available through the study of tissues prepared by simple techniques and that searching cytological studies involving more difficult techniques offer very great promise.

The association of biochemical systems with structural details within cells is a biological goal of greatest importance. Success with a few systems in which vitamins are operative does not seem impossible of achievement and should carry us far toward a conception of the anatomy of life.

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PHYSIOLOGICAL REVIEWS

VOL. 22

OCTOBER, 1942

No. 4

THE APPLICATION OF LABELING AGENTS TO THE STUDY OF PHOSPHOLIPID METABOLISM

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Phospholipids are fatty compounds containing nitrogen and phosphorus. Three molecular types are usually recognized as phospholipids: the lecithins, the cephalins and the sphingomyelins. Other names such as "phospholipins," "phosphatides," "aminophosphatides" and "phosphoaminolipids" have been applied to these compounds, but since the term "phospholipid" is commonly used in this country it will be employed to designate the compounds dealt with in this review.

Several phases of phospholipid metabolism have been reviewed in recent years by Sinclair (1, 2). The question of phospholipid in the transport of fat in the animal body has been studied by Bloor (3). Hevesy has summarized his work with radioactive phosphorus (4). A detailed treatment on the structure of lecithin, cephalin and sphingomyelin has been presented by Working and Andrews (5).

The lecithin molecule may be regarded as formed from 5 primary components: 2 fatty acids, glycerol, choline and phosphoric acid. These are joined by 2 general types of bonds: 1, fatty acid-glycerol ester linkage, and 2, phosphate-alcohol ester bond. Phosphate is bonded in the lecithin molecule by 2 ester linkages, one of which is with glycerol, the other with the base choline. The formation of all these bonds can be followed with suitable tracers. It is necessary to define synthesis or formation of the lecithin molecule with respect to the formation of these bonds. It is inconceivable that all 5 components unite simultaneously to form a new molecule. The various components may or may not be assembled in an orderly sequence in which the formation of a given bond always precedes that of another. If the molecule is assembled in such a way that the formation of a given bond does precede that of another, then the observed rate at which any component is incorporated into a phospholipid molecule will be equal to the rate at which the other components enter the molecule. If the molecule is not assembled in an orderly fashion (as seems most likely), then it is improbable that the reaction rates for the formation of the phosphate-glycerol bond, the fatty acid-glycerol bond and the nitrogen base-phosphate bond will be the same. In the latter case it would be expected that the rate of turnover of phospholipid phosphate differs from that of phospholipid fatty acids and phospholipid nitrogen base. Hence, until it has been shown that the rate of entrance of a given component into the lecithin molecule is the same as the rate of entrance of the other components, the formation of each bond should be considered an independent reaction.

The above reasoning applies not only to lecithin, cephalin and sphingomyelin, but equally well to any compound organic molecule in a biological system. The

type of bonds joining the primary components of cephalin molecules is identical with that of the lecithins. Phosphate is bonded in a cephalin molecule by 2 ester linkages, one with glycerol and the other with the base ethanolamine, or possibly with the amino acid serine. In sphingomyelin there are 2 ester linkages (one between the sphingosine radical and phosphate, the other between the latter and choline) in addition to a peptide link ($\text{NH}-\text{CO}$) between the sphingosine and a fatty acid radical.

Isotopes of phosphorus, nitrogen and hydrogen have been used to study the incorporation of the phosphate radical, the nitrogen base and fatty acids, respectively, into phospholipid molecules. The fatty acid component of the phospholipid molecule has also been labeled by unnatural fats.

I. LABELING OF THE PHOSPHATE RADICAL. A. *General Considerations in the Use of Radioactive Phosphorus.* 1. *Properties of phosphorus isotopes.* The radioactive isotope of phosphorus ^{32}P has been used extensively for labeling of the phosphate radical of the phospholipid molecule. Radiophosphorus is readily prepared by bombardment of P^{31} with deuterons accelerated in the cyclotron. Radioactive P^{32} of very high specific activity $\left(\frac{\text{P}^{32}}{\text{P}^{31}}\right)$ has also been prepared from carbon disulphide that had been subjected to bombardment with fast neutrons. The fact that P^{32} has a relatively long half-life (14.3 days) has added to its usefulness in tracer studies.

Isotopes of an element differ only in mass. Since they have the same electronic configuration and nuclear charge, they have been considered identical chemically. Only properties that depend upon the mass of the atom¹ will differ among isotopes. Such differences are best shown in the hydrogen isotopes $^1\text{H}^1$, $^1\text{H}^2$, and $^1\text{H}^3$; in these the percentage difference in isotope mass is the greatest of all known elements. Some differences in the biochemical behavior of the isotopes of K have been reported (6, 7). It is not possible at present to state what biological difference, if any, exists between the isotopes P^{31} and P^{32} . Certainly it should not be any greater than that observed in the K isotopes. The greatest biochemical difference would obtain for isotopes of an element functioning in the body as a single ion or atom. In the case of phosphorus it is not the mass of atomic phosphorus but rather that of the phosphate radical that should be considered. Since the differences for the K isotopes did not exceed 1 or 2.5 per cent, the differences to be expected between the behavior of P^{31}O_4 and P^{32}O_4 should certainly be less. The greater the weight of the reacting portion of the molecule that contains the isotope, the less will be the effect of differences in isotope mass.

2. *Radiation effects.* In order that a radioactive isotope may serve as an indicator of the endogenous metabolism of its unlabeled isotope within the animal organism, certain precautions must be observed. The radioactivity of the injected dose should have no deleterious effect on any of the tissues. The amount of the substance administered should not unduly increase the content of this substance already present in the organism.

¹ Diffusion rates, reaction rates, molecular velocities, etc., are a function of the molecular mass.

The deleterious effects of x-rays and radium emanations are well established. The more recent types of radiational treatments have employed artificially made radioactive isotopes, the same radioactive substances that are used in tracer studies. There can be no question of the existence of biological effects of atomic radiation from any source, and there is need for more precise knowledge of such effects at the present time. Biological effects of radiation are due to the production of ionization or of agitation by the radiations, i.e., disruption of molecules in their paths. The effectiveness of a radiation in producing damage to a tissue depends upon several factors: 1, total energy of the particles or rays; 2, their mass; 3, their velocity; 4, their charge, and 5, the extent to which they are absorbed by the medium through which they pass and the number of molecules they activate or ionize.

Radioactive phosphorus produces only β -particles in its disintegration. Most other radioactive isotopes have gamma rays of various intensities that are accompanied by showers of β -particles of different energies. Isotopes of this type in common use are Na^{22} , Zn^{69} , I^{131} and Br^{82} . There are no known radioactive isotopes that produce only gamma rays and no beta particles. P^{32} is among the few radioisotopes that produce beta particles but no gamma rays.

The radioactivity of the P^{32} samples used in the Berkeley laboratories as tracers in phospholipid studies is not kept constant; the dose injected is determined by the tissue studied. Thus in the case of the brain, which is relatively inactive in its uptake of P^{32} , a dose corresponding to about 0.05 microcurie per gram body weight is employed. In the case of a more active tissue like the liver, solutions of P^{32} yielding a radioactivity of 0.001 microcurie per gram body weight are administered. Jones (8) has shown that within very wide limits the percentage recovery of total P^{32} and phospholipid P^{32} in tissues of the mouse is not influenced by variations in the radioactivity of P^{32} . Five groups of tumor-bearing mice were injected with a solution of Na_2HPO_4 containing the same amount of P^{31} but differing as regards P^{32} content. The radioactivity varied from 1 to 70 microcuries. In spite of the 70-fold difference in radioactivity of the doses employed, no difference was observed in the percentage of the administered dose recovered² as phospholipid P^{32} or as total P^{32} in liver or tumor. Apparently there is a large factor of safety in the radiational dosage of P^{32} used as tracer.

3. *Phosphate-dose effects.* The amount of labeling agent administered is also of some importance. Its administration should not materially alter the total amount of the agent already present in the animal. A good example of the great care that must be used in the amount of labeled substance injected is

² A Curie of any radioactive material undergoes the same number of disintegrations per unit time as one gram of radium, namely, 3.7×10^{10} disintegrations per second. In the Berkeley laboratories, P^{32} is measured with a Lauritsen electroscope against a uranium X_2 standard, equivalent to one microcurie, i.e. the UX_2 produces 3.7×10^4 primary β particles per second.

³ According to Goudsmit, if radiational effects were involved in the synthesis of a compound, the P^{32} recovered in the compound would be approximately proportional to the square of the P^{32} administered (9).

shown in the study of iodine metabolism with I^{131} . The total amount of iodine in the animal body is small; a normal 200 gram rat may contain as little as 0.05 mgm. of iodine. The introduction of as little as 0.03 mgm. of iodine (60 per cent of that already present in the rat) will flood the organism with iodine, and its distribution may not reflect a true picture of the endogenous iodine metabolism. In the case of radioisotopes it is possible to prepare samples that contain atoms numerous enough for detection by their radioactivity but too few for chemical measurement. By the administration of such a dose of radioiodine it is possible to label the circulating iodine within the animal without altering measurably the amount of iodine already present in the animal. This feature can be obtained at present only by a labeling device as sensitive as that provided by the radioactive isotope.

In the case of Na_2HPO_4 labeled by the inclusion of P^{32} , it has been shown that relatively large amounts can be administered without interfering with its value as a safe labeling agent (10). No difference in the P^{32} recovery in such tissues as brain and blood was observed when such widely differing amounts of phosphorus (Na_2HPO_4 containing P^{32}) as 6.0 and 0.3 mgm. were injected into 200 gram rats. The finding that such large variations in the amount of Na_2HPO_4 injected produced no change in the percentage of the administered labeled phosphorus recovered in brain and blood is not surprising. Although 6 mgm. of phosphorus (27.5 mgm. as Na_2HPO_4) is an appreciable amount, it represents but a small fraction of the total inorganic phosphorus already present in the adult rat; hence its introduction into the rat does not markedly increase the animal's phosphate content.

Radioactive phosphorus was first used as an indicator of phospholipid metabolism in the laboratories of Hevesy and of Artom. This was soon followed by extensive studies at Berkeley. It has been repeatedly shown that phosphate is incorporated into the phospholipid of all tissues examined. The term lipid phosphorylation will be used here to describe the formation of phosphate bonds in the phospholipid molecule.

B. *Quantitative Measures of Phospholipid Turnover with P^{32}* . In some of the early work the phospholipid P^{32} incorporated into a tissue was expressed as a fraction of the administered P^{32} . Two groups of workers (Hevesy and Hahn, 11; Artom *et al.*, 12) have introduced schemes for the determination of the rate of phospholipid turnover in a tissue, as shown by renewal of its phosphate radical. In the following discussion a general treatment of the question of phospholipid turnover will be given, followed by a consideration of the schemes proposed by Hevesy and Artom.

The terms "turnover," "turnover rate," "turnover time," will be used only in the sense defined below. Their definition is made necessary by the confusion of terms now employed in tracer studies.

Phospholipid turnover in this section refers to the process of phospholipid renewal in an organ; this involves the uptake as well as the synthesis of phospholipid and the decomposition as well as the outgo of phospholipid. The term "phospholipid renewal" refers to the renewal of a given component, not

necessarily to the whole molecule. It is only the component that is being labeled whose renewal is measured experimentally.

Phospholipid turnover rate is the percentage of phospholipid present in an organ or tissue that has been turned over (see *Phospholipid Turnover* above) per unit of time during the course of an experiment. To have quantitative significance it may be necessary to take into account the possibility of breakdown of newly formed phospholipid molecules (i.e., labeled molecules formed at the expense of the labeling agent) as well as their loss by way of the bloodstream. Apparently this type of loss and breakdown is disregarded in Hevesy's calculation, which will be described below. He appears to assume that negligible amounts, if any, of the new phospholipid formed during the time of the experiment have left the tissue or decomposed, and that during the course of the experiment breakdown or loss involves only molecules that were present before the labeling process was introduced.

Complete phospholipid turnover time is the time required to form and/or take up (i.e., acquire from some other tissue or tissues by way of the bloodstream) an amount of phospholipid equal to that present in the organ in the steady state.

Some of the considerations involved in a quantitative measure of phospholipid turnover are given below. Unless the assumptions upon which calculations are based are critically evaluated and the proper conditions maintained throughout the experiment, calculated values for rate of phospholipid turnover have little, if any, meaning.

1. *The steady state.* For simplicity, the phospholipid content of every cell, tissue or organ is assumed to remain constant throughout the experiment. This means that the same amount of phospholipid enters and/or is synthesized in the organ as leaves and/or is broken down. In other words, the rate of appearance of phospholipid in the system equals the rate of disappearance and is constant for the duration of the experiment.

2. *Rate-determining step.* The rate-determining step should be known to make valid the calculations for the rate of phospholipid turnover. If equilibrium between plasma inorganic phosphate and the immediate precursor of phospholipid at the site of reaction is instantaneous, then a calculation of the rate of phospholipid turnover based on the measurement of specific activity of plasma inorganic phosphate will be valid. If the rate-determining step is the synthesis of phospholipid from some intermediate, say xP, and if the formation of xP from phosphate of the plasma is the fast step in the reaction, then again a calculation of the rate of phospholipid turnover based on the specific activity of plasma inorganic phosphorus will be valid. This is true because a rapid equilibrium exists between xP and plasma phosphate, so that the specific activity of the intermediate is the same as that of plasma phosphate.

If xP is slowly formed from plasma inorganic phosphate and a fast equilibrium exists between xP and phospholipid, then the rate of phospholipid turnover can also be calculated from the specific activity of plasma inorganic phosphate, since the specific activity of the phospholipid is the same as that of xP.

On the other hand, if the conversion of plasma phosphate to xP and the conversion of the latter to phospholipid are 2 slow reactions, then both reactions influence the rate of phospholipid formation. If this is the case, only information about the specific activity of the immediate precursor xP at the site of reaction will yield valid data for the measurement of phospholipid turnover. In this particular case, the possibility exists that xP is present in a tissue in amounts negligible in comparison with the actual amount turned over per unit of time. This will be the case if xP is present to the extent of 1 unit in the cell and is formed at the rate of 100 units per minute. Hence, even though the conversion of phosphate to xP is one of the slow steps, as assumed above, yet because xP is turned over so rapidly with respect to the amount present, its specific activity reaches instantaneous equilibrium with that of phosphate.

It is not unlikely that we are dealing with more than one intermediate (x_1P , x_2P , etc.) in the synthesis of phospholipid from plasma phosphate. Indeed, one or more intermediates may be connected by slow steps. A further complication in interpreting such a calculation will arise if several different slow paths for phospholipid formation exist, each involving a phosphorus-containing precursor. To make a valid determination of the rate of phospholipid turnover in this latter case, it would be necessary to know the specific activity of the immediate phospholipid precursor of each path and the amount of phospholipid formed by way of each path. The situation is further complicated by the fact that not only are there 3 distinct types of phospholipid, lecithin, cephalin and sphingomyelin, but also each type represents a group of substances differing in the nature of their fatty acid components.

3. *Time interval.* If a single dose is injected, the choice of the interval of the experiment for the calculation of turnover rate should be such that data on the ascending part of the specific activity-time curve are available, since in this time interval synthesis of radiophospholipid rather than breakdown is the predominant factor. This is desirable in order to avoid complicating the calculation. Complications can also be avoided if the specific activity of the immediate precursor is maintained constant at the site of phospholipid formation. Hevesy has employed continuous intravenous injections of phosphate as a means of maintaining a constant phosphate S.A. of the plasma. Whether or not this makes for a similar constancy in the specific activity of the immediate precursor at the site of phospholipid formation is not known.

4. *Specific activity of the immediate precursor at the exact site of phospholipid formation.* In experiments of short duration, however, the rate of transport of the labeling agent to the actual site of formation is an important factor in the amount of labeled substance recovered, since the amount of isotope recovered in the substance under investigation is a direct function of the specific activity of the immediate precursor at the site of formation. If the site of formation is outside the cell, then it is safe to assume that the specific activity of the labeling agent at the site of formation is the same as that of the labeling agent in the surrounding fluid. If the site of formation is inside the cell, then the rate of penetration of the labeling agent into the cell is a factor in the calculation of the

rate of phospholipid synthesis.⁴ It should be stressed that the site of formation need not necessarily involve the whole cell but only a part of it. If the latter is the case, then it is the specific activity of the immediate precursor in this particular part of the cell, not that of the whole cell, that is the decisive factor in determining the amount of isotope or labeled agent recovered in the substance synthesized.

The foregoing indicates some of the difficulties involved in any attempts to arrive at an exact measure of the rate of phospholipid turnover. The obstacles involved may be further shown by the two types of calculations made by Zilver-smit (13), these differing only in a single fundamental assumption. Both methods involve the maintenance of a constant specific activity of the immediate precursor at the site of the reaction. In method I⁵ it is assumed that the newly

⁴ For example, if in a 2-hour experiment one hour is required for the labeling agent to make its appearance at the site of the reaction, then synthesis is being measured only during the second hour of the experiment. If the experiment were extended to 20 hours, then synthesis would be measured for 19 hours and the interval, namely, one hour, required for the transport of the labeling agent to the site of the reaction would be of less significance than in a 2-hour experiment.

⁵ Derivation of the equations used in calculations made by method I and method II (Zilver-smit).

Method I. Let X = counts of phospholipid found at any time in an organ or tissue

Y = total amount of phospholipid phosphorus present in the organ
(assumed to remain constant)

A = specific activity of precursor which is assumed constant at the site
of reaction for the duration of the experiment

k = rate of phospholipid appearance and disappearance (assumed to
remain constant)

$$\text{then } \frac{dX}{dt} = kA - k \frac{X}{Y}$$

$$\int_0^X \frac{dX}{A - \frac{X}{Y}} = k \int_0^t dt$$

$$-Y \ln \left(\frac{A - \frac{X}{Y}}{A} \right) = kt; \quad A - \frac{X}{Y} = A e^{-\frac{k}{Y}t}; \quad \frac{X}{Y} = A(1 - e^{-\frac{k}{Y}t})$$

$$\frac{X}{Y} = 1 - e^{-\frac{k}{Y}t}$$

But at complete turnover time $t = \frac{Y}{k}$; therefore at complete turnover time

$$\frac{\text{specific activity of phospholipid}}{\text{specific activity of precursor}} = \frac{\frac{X}{Y}}{A} = 1 - \frac{1}{e} = 0.63$$

Method II. The same notation as in Method I is used. If at the end of a given time interval X counts of phospholipid are found, the amount of precursor incorporated into the compound during this time interval is $\frac{X}{A}$. If Y is the total amount of phospholipid phos-

labeled phospholipid molecules act as though completely mixed with the old phospholipid molecules already there and that the breakdown of phospholipid (proceeding simultaneously with new formation) occurs at random and involves both old and newly formed molecules. As noted above, the total phospholipid content of a tissue remains constant for the duration of the experiment. In method II⁵ Hevesy's assumption is made, namely, that "with the formation of new phosphatide molecules, the decomposition of an equal or similar number of old molecules goes hand in hand" (11). By method I it can be shown that the specific activity of phospholipid at complete-turnover time (see definition above) is equal to $0.63 \times$ specific activity of the precursor. By method II, however, at complete-turnover time the specific activity of phospholipid equals that of the precursor. The absolute difference in the values obtained for complete-turnover time by these 2 methods of calculation will depend upon the particular set of conditions that prevail during the experiment. It should be noted here that methods for measurement of phospholipid turnover need not involve the maintenance of a constant specific activity of the precursor at the site of reaction. It is possible to determine phospholipid-turnover time from a single injection of labeled phosphate. This type of calculation, however, would still involve assumptions regarding the steady state, the rate-determining steps, and the specific activity of the precursor at the site of the reaction (13).

Although these considerations on the use of tracers for the quantitative measure of turnover are more rigorous than those presented heretofore, they obviously suggest that further work (both theoretical and experimental) is necessary to clarify the interpretation of labeling. It should not be inferred, however, that the early attempts made to derive quantitative measures of phospholipid turnover with P^{32} are not of value.

The Hevesy measurement of the rate of phospholipid turnover (11). By means of ratios of specific activities of phospholipid to specific activities of phosphate, Hevesy has calculated the rate of phospholipid turnover in various tissues of the animal. He maintained a constant specific activity of inorganic phosphorus in plasma by continuous injection of labeled Na_2HPO_4 . He took into consideration the difficulty in determining the exact site of phospholipid formation; for this reason he made calculations for both intracellular and extracellular phosphate.

phorus present in the organ, the fraction of the total amount of the phospholipid present which has been formed during this interval (fraction turned over) is

$$\frac{\frac{X}{A}}{\frac{X}{Y}} = \frac{\frac{X}{Y}}{A} = \frac{\text{S.A. Phospholipid.}}{\text{S.A. Precursor}}$$

Therefore, when $\frac{\frac{X}{Y}}{A} = 1$, an amount equal to the total amount of compound has been synthesized. The time for this to occur is the complete turnover time.

It should be noted here that if random breakdown does occur, Method II can be used only in experiments of short duration since over short intervals breakdown of radiophospholipid can be neglected.

This gave upper and lower limits for the rate of phospholipid turnover. He shows by his calculations that, if phospholipids are synthesized inside the cell, then the liver has a higher rate of phospholipid turnover than the kidney, whereas if phospholipids are formed from extracellular phosphate, then the kidney has a higher rate of phospholipid turnover than the liver (table 31 (11)).

Hevesy states that he measures the rate of phospholipid synthesis from inorganic phosphorus "independent of the actual mechanism involved." As noted above, however, the question of intermediates is important if a quantitative meaning is to be attached to the concept of turnover. This can be further illustrated by the following example:

Let us take the case in which phospholipid is formed in organ A from a P-containing precursor that has been formed in some other organ. Let us assume that this precursor is not in rapid equilibrium with the intracellular phosphate of organ A. Then the specific activity of this precursor is independent of the specific activity of the intracellular phosphate of organ A. Now consider the 2 possibilities: 1, the specific activity of the precursor is greater than that of the intracellular phosphate of organ A. Hevesy would calculate the rate of phospholipid turnover in organ A from
$$\frac{\text{Specific Activity Phospholipid}}{\text{Specific Activity Inorganic P}}.$$
 But this value

would be higher than the true value since the specific activity of the precursor is greater than the specific activity of intracellular phosphate; 2, the specific activity of the precursor is smaller than that of the intracellular phosphate of organ A. Then the value for turnover rate as calculated by Hevesy would be less than the true value. Until the mechanism of phospholipid synthesis (intermediates, rate-determining steps, etc.) is established, the validity of measuring turnover rates from only inorganic phosphorus is open to question.

Mathematical expression of phospholipid turnover as derived by Artom, Sarzana and Segré (12). Their treatment involves assumptions similar to those made for method I above. As a further assumption, they consider the distribution of P^{32} in only skeleton, blood and liver, and neglect the influence of other tissues as well as excretion during the period of measurement. They arbitrarily assume values with respect to 1, the rate at which P^{31} enters and leaves the liver and skeleton, and 2, the relative proportions of P^{31} in bone, blood and liver. On the basis of these assumptions they calculate theoretical specific-activity time-curves for phosphate in blood and skeleton and for phospholipid in liver; from these they attempt a rough comparison of the rates of phospholipid formation in various tissues. Their paper is an interesting contribution to the concept of labeling as a means of measuring the rate of phospholipid turnover; the limitations of their method are well discussed.

C. *Lipid Phosphorylation in the Intact Animal.* In the following sections the incorporation of labeled phosphate into phospholipids in various tissues and organs of the intact animal will be considered.

1. *Liver.* Inorganic phosphorus is rapidly incorporated into phospholipid of the liver (11, 12, 14, 15, 16). Following the injection of a single dose of P^{32} as Na_2HPO_4 , a sharp rise and fall in the content of phospholipid P^{32} are observed

in the liver; maximum concentrations of radiophospholipid occur in the liver of rat and mouse (17) at about 8–10 hours after the injection. This deposition of radiophospholipid in the liver was found to be unimpaired in rats deprived of both kidneys and gastrointestinal tracts (18). According to Hevesy, about 14 to 19 per cent of the liver's phospholipid is renewed in 4 hours if it be assumed that phospholipid formation occurs at the expense of intracellular inorganic phosphorus (11). Chargaff states that lecithin is more rapidly formed in the liver than cephalin (19, 20). Hevesy, however, points out, interestingly enough, that 4 hours after the intravenous injection of labeled phosphate the turnover rate of liver cephalin exceeds that of lecithin, whereas in experiments lasting one day or more liver lecithin is renewed more rapidly than cephalin (11). According to Hunter (21), sphingomyelin P^{32} accounts for only a small part of the phospholipid P^{32} deposited in the liver.

2. *Phospholipid turnover of the liver and lipotropic action.* Radioactive phosphorus has been used to explain the mechanism whereby choline and other lipotropic substance prevent and cure fatty livers. Choline (22), betaine (23) and methionine (24), all of which have been shown to inhibit the infiltration of abnormal amounts of lipids in the liver, stimulate phospholipid turnover as measured by the incorporation of injected P^{32} into phospholipids. The feeding of diets rich in cholesterol (25), which produce fatty livers, depressed this incorporation in the liver. Thus 2 processes, the entrance of fat into the liver and its release from the liver, have been linked with the rate of lipid phosphorylation.

The stimulating effect of a single dose of choline on lipid phosphorylation is of short duration (22). An increase in phospholipid activity was observed about one hour after choline ingestion, but its effect is no longer demonstrable 10 hours later. The effect of choline upon phospholipid metabolism depends upon the amount fed; between the limits of 0 and 30 mgm. the extent of phospholipid stimulation increased with the amounts fed. The significance of this effect of choline in stimulating the rate of lipid phosphorylation has at times been confused with the demonstration that labeled choline enters the phospholipid molecule. Stetten (28) has clearly demonstrated that administered labeled choline finds its way into the phospholipid molecule. Welch (26, 27) found that administered arsenocholine is incorporated into lecithin. The observations of Stetten and Welch, however, give no information about the comparative rates of lipid phosphorylation before and after choline treatments (22).

It is of interest to note here that the effect of choline upon the rate of lipid phosphorylation was observed despite the fact that the content of total phospholipid in the liver showed no measurable change. A relative constancy in the total phospholipid content of the liver should therefore not be taken as evidence of phospholipid inactivity. The significance of total-phospholipid content of the liver as an index of phospholipid activity or turnover has been considered in some detail elsewhere (25).

Choline has a more pronounced lipotropic action than betaine (27, 30); in small doses betaine is less effective than choline in stimulating lipid phosphorylation. This lesser effectiveness of betaine than of choline has been explained by

Stetten (28) as due to the fate of betaine in the animal body. Isotopic betaine containing heavy nitrogen was fed to rats; the choline isolated was poor in N^{15} , whereas glycine was rich in isotope. Betaine is therefore demethylated to glycine. Apparently only the methyl groups of betaine, and not the rest of the molecule, are used for choline synthesis.

Methionine, cystine, cysteine stimulate lipid phosphorylation (24). Only the first of these has a lipotropic effect. Glycine, alanine, serine, tyrosine, proline, glutamic acid and asparagine have been shown to have no effect on the rate of phospholipid turnover as measured with radioactive phosphorus (31).

3. *Blood.* Administered P^{32} appears rapidly in phospholipid of the plasma. This has been shown by Hevesy and Hahn (11) in experiments in which they maintained a constant level of labeled inorganic phosphorus in plasma of rabbits for 9 days and by Fishler (32) in experiments with single injections of P^{32} in dogs. As early as 3 hours after a single injection of P^{32} , appreciable amounts of radiophospholipid are already present in plasma of the dog. By 40 hours, 0.5-1 per cent of the administered P^{32} is incorporated into phospholipid of the dog's total plasma (32).

The incorporation of administered P^{32} into phospholipid is a much slower process in corpuscles than in plasma (11, 32, 33). Following the injection of a single dose of radiophosphorus in the dog, there occurs a sharp rise in the phospholipid P^{32} content of the plasma, with the maximum by 36 hours; it required more than 2 days for measurable amounts of phospholipid P^{32} to make their appearance in the dog's cells (32). At no time do the cells attain a phospholipid P^{32} content greater than 25 per cent of the maximum found in plasma (32). In contrast to plasma, cellular radiophospholipid leaves the blood slowly (32). The highest amounts of radiophospholipid in cells appeared between 200 and 300 hours; at 500 hours about one half of that amount was still present in the cells (32). Since the phospholipid contents of cells and plasma are roughly the same and do not change during the course of the experiment, the above values may be considered as indicative of the relative specific activities of corpuscle and plasma phospholipid.

According to Hevesy and Aten, the renewal of corpuscle phospholipid in a period of 28 hours is only one-third of that in the plasma of the laying bird (33). In man, the specific activity of phospholipid phosphorus in corpuscles reaches a value about one-half of that of the plasma as late as 8 days (33).

Plasma phospholipids do not penetrate corpuscles readily (11, 32, 33, 34). Hahn and Hevesy (34) injected P^{32} into a rabbit and separated its plasma; this plasma, containing radiophospholipid, was then mixed with corpuscles removed from another rabbit. At the end of 4.5 hours of incubation only 5 per cent of the phospholipid present in corpuscles had exchanged. The results obtained *in vivo* are equally striking; 37 hours after the injection of P^{32} into the dog, Fishler (32) found that the phospholipid P^{32} content per cubic centimeter of plasma was more than 10 times that of the corpuscles. Although large amounts of radiophospholipid were present in the plasma at the 12-hour interval, practically no phospholipid P^{32} was observed in corpuscles at this time despite the fact that

they already contained considerable amounts of P^{32} . Even as late as 60 hours, a marked difference between phospholipid P^{32} content of corpuscles and plasma was still observed (32). These observations are not incompatible with the view that lipid phosphorylation within the corpuscle occurs mainly at the time it is formed.

Labeled phospholipid introduced intravenously into animals disappears rapidly from the plasma (35, 36). Liver and spleen are most active in their removal.

4. *Liver as the site of phosphorylation for plasma phospholipid.* Comparisons of the specific activity of phospholipid phosphorus of plasma with that of the phospholipid phosphorus of organs have led Hevesy (11, 33) and Artom (37) to infer that the incorporation of phosphate into phospholipid molecules of plasma occurs largely in the liver. Both groups of workers, however, are careful not to exclude other organs. A study of this point has been made by Fishler (32), who compared the phospholipid P^{32} per gram of total phospholipid in plasma, liver, small intestine and muscle at 6, 18, 36 and 98 hours after the injection of inorganic P^{32} into dogs. At the first interval, the phospholipid P^{32} per gram of phospholipid in the liver was higher than in any other tissue. At the 18-hour interval the values in liver and plasma were the same, and in these 2 tissues the phospholipid P^{32} per gram of total phospholipid was higher than in small intestine, kidney or muscle. At 36 hours, values for plasma and liver were still the same and higher than those in the other tissues. Not before 98 hours did the values for kidney and small intestine approach those for liver or plasma (32). The close agreement in the values for phospholipid P^{32} per gram of total phospholipid in liver and plasma between 18 and 98 hours suggested a close tie-up between these 2 tissues. This observation has led to a study of lipid phosphorylation in the hepatectomized dog (32). Immediately after removal of the liver, dogs were injected intravenously with inorganic P^{32} . Practically no phospholipid P^{32} was recovered in plasma as late as 3-6 hours after extirpation of the liver; at these times 0.4 per cent of the injected P^{32} had been incorporated into phospholipid of both kidneys and about an equal amount in the whole small intestine. These values for kidney and small intestine were approximately the same as those obtained for these tissues in normal dogs. This observation is certainly in line with the view that the liver is the main (and possibly the only) site for phosphorylation of plasma phospholipid. It should be noted here that the site of phosphorylation need not coincide with the site of esterification of the glycerol component with fatty acids.

Intestine. Radiophospholipid is rapidly deposited in the gastrointestinal tract after the injection of P^{32} (11, 12, 15, 16, 18, 19). The phospholipid activity of its various parts is not the same; the small intestine is very much more active in lipid phosphorylation than stomach or large intestine (18). In the bird the deposition of injected inorganic P^{32} as phospholipid is greater in small intestine than in gizzard, proventriculus, ceca or colon (16). Artom *et al.* (12) state that the specific activity of phospholipid phosphorus in the intestine exceeds that in the liver when the P^{32} is administered orally. Fries *et al.* (18) have also ob-

served that the recovery of phospholipid P^{32} in the small intestine is greater when P^{32} is administered *per os* than when it is administered subcutaneously. The presence of fat in the gastrointestinal tract increases the deposition of radiophospholipid in the small intestine (12, 18). At early intervals (4 hours) after P^{32} administration a more rapid renewal of mucosal cephalin than of lecithin has been observed (11); at later intervals, however, the incorporation of P^{32} into phospholipid is apparently greater in lecithin than in cephalin of the intestine (19).

6. *Kidney.* In the intact animal administered phosphate is readily incorporated into kidney phospholipid. Following a single injection of P^{32} , the rate of deposition and of disappearance of labeled phospholipid is slower in kidney than in liver or small intestine (14, 15). Kidney has also been compared with other tissues in respect to the specific activity of phospholipid phosphorus (i.e., the ratio of phospholipid P^{32} to phospholipid P^{31}). According to Artom *et al.* (12, 14, 43), the specific activity of kidney phospholipid is lower than that of small intestine and liver phospholipid at the early intervals after P^{32} administration in the rat. Fishler *et al.* (32) measured the deposition of phospholipid P^{32} per gram of phospholipid in the kidney, liver and small intestine of the dog at 6, 18, 36 and 98 hours after the intraperitoneal injection of P^{32} . At the first time-interval, the values for kidney were lower than those for liver but equal to or higher than those for the small intestine. At 18 and 36 hours the specific activities of the kidney phospholipids remained lower than those of the liver and about equal to those of the small intestine. At 98 hours the specific activities of phospholipid in all 3 tissues were roughly the same.

That kidney *can* phosphorylate fat has been established by *in vitro* experiments with surviving slices of this tissue (see below). That lipid phosphorylation *does* take place in the intact kidney has been demonstrated with the aid of the hepatectomized dog (32). Despite the fact that no phospholipid P^{32} was found in the plasma of the liverless dog, its kidneys contained radiophospholipid in amounts similar to those found in normal dogs.

Hevesy and Hahn point out that inorganic phosphorus enters the kidney cells very rapidly from the bloodstream and for this reason it is not safe to infer merely from the rate at which injected inorganic P^{32} is converted to phospholipid P^{32} that the turnover of phospholipid by the kidney is a rapid process (11). These workers have employed the specific activities of both inorganic phosphorus and phospholipid phosphorus as a measure of the rate of phospholipid turnover in organs. When turnover rate was calculated on the assumption that phospholipid formation occurs at the expense of intracellular inorganic phosphorus, the turnover of kidney phospholipid was much slower than that of either liver or small intestine phospholipid. When it was assumed that phospholipid formation involved incorporation of extracellular inorganic P, the phospholipid turnover of kidney did not differ markedly from that of either small intestine or liver.

Weissberger found that acidosis induced by the ingestion of NH_4Cl increases the turnover of kidney phospholipid as measured with radioactive phosphorus

(44). The relation of the phospholipid metabolism of kidney to acid-base regulation is pointed out.

7. *Brain*. The incorporation of P^{32} into brain phospholipid of the *intact* animal has been shown by many workers to be a slow process (11, 12, 19, 20, 45, 46, 47, 48). This may be explained by the slow penetration of labeled phosphate into the brain as a whole as well as into its extracellular spaces (11, 48, 49, 50, 51). A progressive increase in the content of radiophospholipid was observed for as long as 200 hours after the administration of inorganic P^{32} (46). Once the maximum amount of labeled phospholipid has been deposited, its loss from the brain occurs very slowly (46). Even as late as 800 hours after P^{32} administration the brain still contained 70 per cent of the amount of labeled phospholipid present at the 200-hour interval. Brain cephalin and sphingomyelin are more rapidly renewed than lecithin (11, 20).

A larger fraction of the administered P^{32} is recovered as radiophospholipid in the various brain divisions of the young than in those of the old animal. Maximum recoveries of injected P^{32} were observed in the whole forebrain of the 15 gram (1 week) rat and in the whole spinal cord, whole cerebellum and whole medulla of the 25 gram (2 weeks) rat (47, 48). An interesting correlation was observed between these recoveries of P^{32} as phospholipid and the rate of deposition of total phospholipid in all brain divisions. The maximum deposition of total phospholipid in all parts of the brain (52) occurs in rats between the ages of 1 week (15 gram) and a little over 2 weeks (30 grams). This latter interval represents the period of intense myelination in the brain (53).

Within the same animal the recoveries of P^{32} as phospholipid per gram of tissue are not uniform in the various divisions of the brain (47). The highest recovery of phospholipid P^{32} was found per gram of spinal cord from birth until the time the rat attained a weight of 50 grams. During this time the recovery of P^{32} as phospholipid in spinal cord is 2 or more times that in forebrain. The cord is most closely approached in recovery by medulla, while cerebellum lies between medulla and forebrain. In rats larger than 50 grams a change in the order of phospholipid P^{32} incorporation occurred. In older rats a higher recovery was observed in cerebellum, medulla and forebrain than in the spinal cord, in the order given. For each brain division the P^{32} incorporated into phospholipid can also be expressed as a fraction of its total phospholipid content (specific activities). The highest values for specific activity of phospholipid were observed in the brains of the new-born rats. At all ages the highest specific activity of phospholipid was found in the cerebellum (54).

The slow incorporation of P^{32} into brain phospholipid as compared with that into liver and small intestine raised the interesting question whether nervous tissue could phosphorylate fat independently or whether it derives its phospholipid from plasma. To answer this question a study was made of the formation of phospholipid by surviving brain slices kept in a Ringer-bicarbonate solution containing radioactive phosphorus (55). The ability of brain of both old and young animals to form phospholipid independently was demonstrated. The excised nerve of a dog was also shown to be active in converting phosphate to phospholipid. The *in vitro* formation of phospholipid will be dealt with below.

8. *Muscle.* As compared with its speed in tissues such as liver and small intestine, administered P^{32} makes its appearance as phospholipid P^{32} slowly in skeletal muscle (4, 11, 16, 17). This may be related to the rate of entrance of inorganic phosphorus into the muscle cell (11, 50, 59). No difference was observed in the phospholipid P^{32} contents of leg and breast muscle of the bird at 6 and 12 hours after the injection of labeled phosphate (16). Cardiac muscle incorporates more of the injected P^{32} into phospholipid than does skeletal muscle (16).

The turnover of phospholipid phosphorus is increased in the muscle of rats maintained on a diet deficient in fat (60). Denervation increased the rate at which administered P^{32} is deposited in muscle phospholipid (37, 61). When compared with the corresponding muscle of the intact side, the denervated muscle may show increases of over 200 per cent in the amount of phospholipid P^{32} incorporated. This change appears before any appreciable atrophy of the muscle.

9. *Neoplastic tissues.* Phospholipid metabolism of tumors as measured with P^{32} presents several unique characteristics. Their activity in converting injected inorganic P^{32} into phospholipid is relatively high as compared with that of such tissues as liver, kidney and intestine (4, 17, 62). In contrast to the latter tissues, however, maximum deposition of phospholipid P^{32} in neoplastic tissues may last from 10 to 50 hours (17); the highest concentration of phospholipid P^{32} in liver was observed about 10 hours after P^{32} administration. Haven has measured the turnover of lecithin, cephalin and sphingomyelin in carcinosarcoma 256 (62, 63); the turnover of lecithins was found somewhat more rapid than that of cephalins.

The rate at which injected P^{32} is converted to phospholipid P^{32} is not the same in all neoplastic tissues. Thus lymphosarcoma and mammary carcinoma were found distinctly more active in this respect than sarcoma 180 and lymphoma (17). This individuality among tumors was demonstrated by a procedure in which 2 or 3 types of tumor transplants were grown side by side in the same animal (64). No relation was observed between the phospholipid P^{32} content per gram of tumor and its preceding growth rate as measured by increase in mass (17, 64).

10. *Lipid phosphorylation in the laying bird and in the egg.* The laying bird is characterized by a high phosphorus-turnover. A single 60 gram (chicken) egg may contain as much as 0.5 gram of phosphorus combined in various compounds. Egg-laying increases the rate at which injected inorganic P^{32} is deposited as phospholipid in 3 tissues of the bird: blood, ovary and oviduct (16). Administered P^{32} makes its appearance rapidly in the phospholipid and other phosphorus compounds of the yolk (16, 65). The amount of P^{32} deposited in the yolk can be accounted for as a function of 2 variables: yolk growth and P^{32} availability during the period of new formation (66). From a comparison of the specific activities of various tissues in the laying bird, Hevesy and Hahn (65) have concluded that the bulk of the yolk phospholipid is derived from plasma but formed in the liver. Chargaff (67) states that the rates of formation of "free" lecithin and cephalin and of the "combined" phospholipid accompanying the vitellin fraction are the same. According to Hevesy *et al.* (68), the phos-

pholipid molecules of the chick embryo are not derived from the yolk but are synthesized by the embryo itself.

11. *The adrenal gland and phospholipid formation.* Since it was first proposed by Verzar (38) that adrenalectomy interferes with the processes of phosphorylation, numerous attempts have been made to obtain evidence for or against this view. Stillman (39) measured phosphorylation of fat directly by means of the incorporation of P^{32} into phospholipids of liver and small intestine of rats completely deprived of both adrenal glands. No interference in lipid phosphorylation was observed in adrenalectomized rats no matter whether they were in good condition while maintained on a high NaCl intake or whether they were showing manifestations of adrenal insufficiency. Barnes *et al.* (41, 42) found no interference in the rates of incorporation of tagged fatty acids into phospholipid molecule after adrenalectomy. There are 3 bonds that must be formed in order to build a lecithin or cephalin molecule from its 5 components: 1, fatty acid-glycerol ester linkage; 2, a bond between phosphate and glycerol, and 3, a bond between phosphate and choline. The first of these has been shown to occur in the adrenalectomized animal by means of a tagged fat, whereas studies with P^{32} show that the 2 phosphate bonds can be formed in the absence of the adrenal glands. These 2 types of investigations establish the ability of the adrenalectomized animal to synthesize new phospholipid molecules.

12. *Phlorhizin.* In the intact animal, Weissberger (56) has shown that phlorhizin does not inhibit the incorporation of P^{32} into phospholipid of kidney, intestine, liver, etc. She employed doses as high as 65 mgm. as a single injection. This observation in the intact rat has been confirmed in the writer's laboratory when even larger doses of phlorhizin were used (57). Taurog found, however, that the incorporation of P^{32} into phospholipid of surviving liver slices is decreased in the presence of 0.01 M phlorhizin (58).

D. *The In Vitro Formation of Phospholipid.* The demonstration of the incorporation of phosphate into the phospholipid molecule by excised tissue slices offers a new approach to the study of the mechanism of formation of phospholipid molecules (69). This was made possible by the use of radioactive phosphorus as a labeling device. The sensitivity of the radioactive procedure for measuring phospholipid is such as to permit the accurate determination of traces of newly formed phospholipid. Detection of this type of phosphorylation could only be made with a tagged phosphate, since formation of the new molecules proceeds in the presence of a net decrease in total phospholipid in the system. Although the breakdown of non-radioactive phospholipid does not obscure the entrance of phosphate into the phospholipid molecule, it may, by diluting the P^{32} , decrease the percentage of P^{32} incorporated into phospholipid. Phospholipid formation was first shown for liver slices by suspending them in a Ringer-bicarbonate buffer containing radioactive Na_2HPO_4 of very high specific activity. Since the ratio of inorganic P^{32} to phospholipid P^{32} was kept higher in this type of experiment than in that with intact animals, special precautions were necessary for the separation of phospholipid from labeled phosphate. The formation of radiophospholipid was observed as early as 1 hour, and the amount

formed increased with time up to 4 hours. At the 4-hour interval as much as 6 per cent of the added P^{32} was incorporated into phospholipid per gram of wet tissue when 300 mgm. of liver slices were suspended in 5 cc. of the buffer solution containing 0.18 mgm. of labeled Na_2HPO_4 . The extent to which the separation between phospholipid and phosphate was effected is well shown by zero-time experiments. When no time was allowed for the synthesis to occur, practically no counts (radioactivity) were obtained in the phospholipid extracted despite the fact that the radioactivity of the Na_2HPO_4 present in the bath was equivalent to 1×10^6 counts (69).

Thermodynamic considerations. The nature of the linkages in the phospholipid molecule is such that the hydrolysis of this molecule would be expected to occur with a decrease in free energy. For example, the hydrolysis of the phosphate-alcohol bond in glycerolphosphate involves a value of ΔF° of -2280 calories (70, 71), and it may be expected that the value for the hydrolysis of a similar bond in the phospholipid molecule would not differ greatly. Since there are 2 alcohol-phosphate bonds (both involving the same P atom) in the phospholipid molecule, the hydrolysis of both these bonds in a single reaction should involve a ΔF° value at least twice that noted above for a single bond. The ΔF° for the hydrolysis of the phospholipid molecule to inorganic phosphate would therefore be expected to be of the order of -5000 calories.

In applying these considerations to conditions in the tissue slice, it should be noted that the system is heterogeneous and one in which the concentrations of the various components involved in phospholipid formation are unknown. But the probability remains that even under the conditions of the tissue slice the hydrolysis of phospholipid to inorganic phosphate proceeds with a sizable decrease in free energy and that the tendency of the reaction is far in the direction of hydrolysis. It is to be expected, therefore, that the reverse reaction, namely, the formation of phospholipid from inorganic phosphate, would be an energy-consuming reaction. Hence, in the synthesis of phospholipid from inorganic phosphate coupling with energy-producing reactions should be suspected. Considerable evidence is at hand to show that such coupling actually exists. Anaerobic conditions, cyanide, and other respiratory inhibitors were found to inhibit the incorporation of radioactive inorganic phosphate into phospholipid (72). Apparently phospholipid formation is dependent upon tissue respiration and energy-producing reactions.

Relation of cellular oxidations to in-vitro phospholipid formation. The dependence of phospholipid formation upon oxygen consumption can be shown by measuring the incorporation of labeled phosphate into phospholipid by surviving liver and kidney slices in the presence of various mixtures of oxygen and nitrogen (72). In a nitrogen atmosphere, phospholipid formation by kidney and liver was inhibited to the extent of 90 per cent. Since in this experiment there was no certainty that oxygen was completely excluded from the cells proper, the residual synthesis of phospholipid should not be ascribed to a non-oxygen-utilizing system.

As noted above, energy must be supplied in order to incorporate inorganic

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D. *The In Vitro Formation of Phospholipid.* The demonstration of the incorporation of phosphate into the phospholipid molecule by excised tissue slices offers a new approach to the study of the mechanism of formation of phospholipid molecules (69). This was made possible by the use of radioactive phosphorus as a labeling device. The sensitivity of the radioactive procedure for measuring phospholipid is such as to permit the accurate determination of traces of newly formed phospholipid. Detection of this type of phosphorylation could only be made with a tagged phosphate, since formation of the new molecules proceeds in the presence of a net decrease in total phospholipid in the system. Although the breakdown of non-radioactive phospholipid does not obscure the entrance of phosphate into the phospholipid molecule, it may, by diluting the P^{32} , decrease the percentage of P^{32} incorporated into phospholipid. Phospholipid formation was first shown for liver slices by suspending them in a Ringer-bicarbonate buffer containing radioactive Na_2HPO_4 of very high specific activity. Since the ratio of inorganic P^{32} to phospholipid P^{32} was kept higher in this type of experiment than in that with intact animals, special precautions were necessary for the separation of phospholipid from labeled phosphate. The formation of radiophospholipid was observed as early as 1 hour, and the amount

nience will be grouped under the category of "exchange reactions." For the purpose of this review, "exchange" will be defined as the simple interchange of atoms or radicals between two different molecules, such as the interchange of the phosphate radical between inorganic phosphate and the phospholipid molecule. To be classified as an exchange reaction, interchange must take place without the addition of energy from an external source or from energy-producing reactions.⁶ If, for example, the decomposition of phospholipid molecules to inorganic phosphorus proceeds as a spontaneous reaction at the same time that inorganic phosphate is being reformed into phospholipid with the aid of an energy-producing reaction in the cell, such a process will not be included among exchange reactions as defined here.

Two mechanisms of exchange reactions that conceivably may be involved in the conversion of radioactive inorganic phosphorus to phospholipid will be considered. a. *Exchange through collision*: Collision between an inorganic phosphate molecule and a phospholipid molecule could result in an interchange of P atoms or of phosphate radicals. Radioactive inorganic phosphate might thus become incorporated into a phospholipid molecule solely as the result of such a collision. The experimental observations show, however, that the formation of radioactive phospholipid does not take place through such a mechanism. The failure of homogenized liver to form radioactive phospholipid as well as the inhibitory effect of anaerobic conditions and respiratory inhibitors offers convincing evidence that exchange through collision does not occur to an appreciable extent. b. *Exchange due to reversibility of reactions*: A single chemical reaction is usually considered as the net result of 2 opposing reactions proceeding simultaneously in opposite directions at different rates. The rate of the forward reaction usually decreases more and more as equilibrium is approached, whereas the rate of the backward reaction constantly increases as equilibrium is approached. At equilibrium both rates become equal. Even though the tendency of a reaction is far in the direction of decomposition or hydrolysis and the rate at which its equilibrium is attained is slow (this is most likely the case in the decomposition of phospholipid in a tissue-slice experiment), the reverse reaction may occur to a slight degree even during the early period of the forward reaction. This small amount of reversibility gives rise to a possible mechanism of exchange. For example, in the tissue-slice experiment a slow *net* decrease in the total amount of phospholipid was observed (72). Although the rate of decomposition of phospholipid was quite slow (10-20 per cent in 3 hrs.) and equilibrium for this reaction is probably far in the direction of complete hydrolysis, it is still possible that a small but appreciable amount of radioactive inorganic phosphorus becomes converted to phospholipid through the slight reversibility of the reaction. A slight reversibility of this kind must be kept in mind in view of the sensitivity of the labeling procedure with radioactive material.

This type of exchange, however, can be excluded as a result of experiments

⁶ This excludes activation energy. Energy of activation may be involved in exchange reactions.

with respiratory inhibitors (72). As already noted, the formation of radioactive phospholipid in the liver is almost completely inhibited under anaerobic conditions and in the presence of cyanide, whereas the decomposition is unimpaired under these conditions. If the formation of radioactive phospholipid actually took place through such an exchange mechanism, it is difficult to see how the rate of formation could be inhibited under exactly the same conditions in which breakdown was unchanged. Moreover, in the case of homogenized liver, formation of radioactive phospholipid was completely inhibited, whereas the breakdown of phospholipid proceeded as usual.

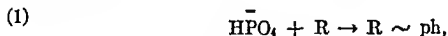
Thus the available evidence indicates that radioactive phosphorus is not incorporated into phospholipid through exchange mechanisms as defined here. The evidence suggests rather that coupling with some energy-producing system of the cell is involved.

2. *Intermediates.* Although it is not possible at present to identify the intermediate compounds involved in the conversion of phosphate to phospholipid, it is of interest to consider here a few of the possibilities. The structure of lecithin suggests that glycerolphosphate and phosphoryl choline might act as intermediates, whereas the structure of cephalin suggests glycerolphosphate and phosphoryl ethanolamine as intermediates. Glycerolphosphate and phosphoryl choline containing P^{32} were prepared in this laboratory by Taurog (58), and their incorporation into phospholipid of liver and kidney was tested *in vitro* by surviving slices of liver and kidney, and also *in vivo*. The incorporation of the P^{32} into phospholipid of liver and kidney in both the intact animal and in slices was demonstrated. These experiments provided no certainty that breakdown of these labeled compounds to inorganic phosphorus did not occur before the conversion of the radioactive phosphorus to phospholipid. It has been shown, however, that the breakdown of glycerolphosphate in the presence of animal phosphatase is very slow at pH 7.4 (75). Moreover, Welch states that the hypothesis that choline chloride is phosphorylated and used in the synthesis of lecithin is supported by the finding that the phosphoric acid ester of choline chloride is unaffected by liver phosphatases and therefore protected from destruction in the liver (27). Although no attempt was made in Taurog's experiments to separate lecithin and cephalin, it should not be inferred that the precursors are the same for these 2 compounds. For example, phosphoryl choline might serve as a precursor for lecithin, but from Stetten's work it is unlikely that it is a precursor of cephalin (28).

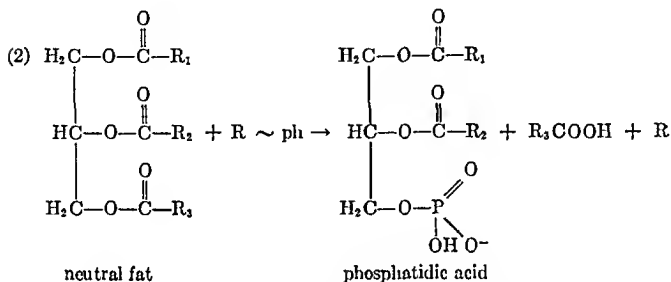
It is very improbable that a single pathway exists for the conversion of inorganic phosphate to phospholipid. It is conceivable that compounds like glycerolphosphate, phosphoryl choline, phosphorylaminoethanol, diglycerides, triglycerides (neutral fat) may be involved as intermediates. The sequence in which various intermediates combine with one another need not be orderly. The possibility that phosphoproteins may serve as intermediates in the formation of phospholipid has been suggested by Chargaff (67).

Lipmann's interesting views (71) on the rôle of "energy-rich phosphate bonds" in intermediary metabolism raise the question whether a compound

containing such an "energy-rich phosphate bond" is involved in the formation of phospholipid. On this assumption a mechanism such as the following may be postulated:



where \sim denotes an energy-rich bond (71)



The addition of choline or ethanolamine to the phosphatidic acid would then complete the phospholipid molecule. The nitrogenous base may also be attached to the compound $\text{R} \sim \text{ph}$ before it enters the phospholipid molecule. Other mechanisms involving $\text{R} \sim \text{ph}$ as an intermediate can be postulated. The oxidative energy required for phosphorylation of lipids might all be used to provide compounds containing energy-rich phosphate bonds. Once such compounds are formed, the phosphorylation of lipids could take place spontaneously. Under anaerobic conditions or in the presence of respiratory inhibitors no $\text{R} \sim \text{ph}$ is formed and the only lipid phosphorylation that can occur is that involving $\text{R} \sim \text{ph}$ already present.

II. LABELING OF THE PHOSPHOLIPID FATTY ACIDS. The complexity of the fatty-acid composition of phospholipid molecules has not made easy the measurement of their fatty-acid turnover. In much of the work that has appeared on this phase of phospholipid metabolism it has been overlooked that a labeling agent serves as a tag only for those substances from which it is chemically indistinguishable. A suitable labeling procedure for studying the endogenous metabolism of fatty acids would be to introduce a *small* amount of fatty acids tagged in such a way that the body cannot distinguish them from its natural fatty acids. The administration of the various naturally-occurring fatty acids labeled by the presence of deuterium in a non-reactive position would approach this closely.

The following methods of labeling fatty acids have been used in phospholipid investigations: highly unsaturated fatty acids, iodized fatty acids, claidic acid, deuterio-fatty acids and conjugated fatty acids. Only the last 3 will be considered here.

1. *Elaidic acid*. This stereoisomer (*trans* isomer) of oleic acid, which does

not occur naturally in the animal body, was introduced by Sinclair as a label for phospholipid fatty acids (76). Elaidic acid is well tolerated by rats even when fed in large amounts. Kohl has shown that this fatty acid is utilized and stored; storage occurs chiefly in the adipose tissue (77, 78). After being deposited in the depots of the rat, elaidic acid is slow to disappear from the body (79). Kohl observed that absorbed elaidic acid disappeared from the body at the rate of 72 mgm. per hour in rats fed elaidin alone and at the rate of 35 mgm. in rats fed elaidin along with sugar and protein (77, 78). Whether or not elaidic acid is transformed into other fatty acids by saturation, desaturation, etc., has not been determined.

The extent to which elaidic acid can be incorporated into phospholipid molecules has been measured by Sinclair. Elaidic acid constituted from 26 to 30 per cent of the phospholipid fatty acids of liver, small intestine, muscle, kidney and blood cells of rats that had been raised on a diet rich in elaidin or fed this fat for long periods of time (2, 80). According to Sinclair, most of the elaidic acid incorporated into phospholipids serves to replace fully saturated fatty acids.

Two tissues fail to incorporate elaidic acid into their phospholipids to any appreciable extent. In rats reared on a high elaidin diet maximum incorporation of elaidic acid into phospholipid fatty acids of the brain was about 7 per cent (81). Even less was found for testes phospholipids (80).

Despite the fact that the maximum amounts of elaidic acid incorporated into the phospholipid fatty acids of small intestine, kidney, liver and muscle were roughly the same, the rate of its entrance into the phospholipids of these tissues differed considerably. Maximum incorporation occurs in less than one day in small intestine and in somewhat over one day in liver. In kidney and muscle one-half of the maximum incorporation occurred in about 3 days (82). Although the maximum incorporation of elaidic acid is somewhat higher in liver lecithins than in cephalins, the rates of uptake by these 2 fractions in the liver are about the same (83).

Administered elaidic acid appears early in plasma phospholipids of the cat (84). A rapid replacement of phospholipid fatty acids of the plasma is indicated by the observation that, in the short space of 8 hours after the feeding of elaidin, about 20 per cent of the plasma phospholipid fatty acids consisted of elaidic acid; more than 20 per cent was found at 48 hours. No elaidic acid appeared in the phospholipids of the red blood cells during the first few days after the feeding of elaidin (84).

Haven (85) has shown that 18 per cent of elaidic acid can be incorporated into tumor (carcinosarcoma 256) phospholipid fatty acids. Little difference was observed in the maximum incorporation in these tumors whether the rats in which they were grown had been raised on a high elaidin diet or had been placed on this diet at the time of inoculation. The rate of entrance of elaidic acid into the phospholipids of this tumor is slower than into those of liver but faster than into those of muscle.

The significance of elaidic acid as a labeling agent will now be considered. It must be obvious that elaidic acid is not a label for all fatty acids of phospho-

lipid molecules. No single fatty acid can serve this purpose. This might explain the finding that not more than 30 per cent of the phospholipid fatty acids of active tissues like liver and small intestine can be replaced by elaidic acid even after the animal has been maintained on a high elaidin diet for many months. The rate at which elaidic acid enters tissue phospholipid would be more meaningful if it were known whether elaidic acid remains unmodified by desaturation or saturation before entering phospholipid molecules. Elaidic acid may not be a suitable labeling agent for studying metabolic changes in fatty acids, since such changes might transform elaidic acid so as to render it indistinguishable from other fatty acids. Fatty acids labeled with deuterium are probably more suitable for this purpose. The effect of permeability of a tissue to elaidic acid upon its rate of incorporation into phospholipid in short experiments should be considered; the question of permeability is of importance as a possible rate-determining step in the use of all labeling agents. The difficulty in accepting a single fatty acid as typical of the fate of all fatty acids is perhaps well brought out by the question of permeability of the cell to different fatty acids. It cannot be inferred that a given cell shows the same permeability to elaidic acid or its derivatives as it does to natural fats. Finally, it has been pointed out that it is not yet known whether one or 2 elaidic acid molecules enter into each phospholipid molecule.

Hevesy points out (11) that in order to obtain quantitative conclusions from experiments carried out with elaidic acid as an indicator it is necessary to compare the elaidic-acid content of the phospholipid in an organ with the elaidic-acid content of the fatty-acid mixture available for phospholipid synthesis in cells of that organ. The assumptions found necessary in order to derive quantitative measures of phospholipid turnover with P^{32} as a labeling agent apply equally well to any other labeling agent.

2. *Fatty acids with a characteristic absorption-spectrum.* Miller and Burr (86) have used tung oil which contains eleostearic acid with 3 conjugated double bonds. More recently Miller *et al.* (87) used prolonged saponification to convert fatty acids of low ultraviolet absorption to ones with conjugated bonds having high spectral absorption. In this way the linoleic acid of corn oil is converted to fatty acid containing conjugated double bonds, a process that results in an increase of approximately 100 times in the absorption coefficient (88). The use of this labeling agent is very limited. Experiments must be confined to short intervals, since the labeled fatty acid is probably changed in the animal body to substances with low ultraviolet absorption. It is apparent that results obtained with fatty acids labeled by conjugation can yield interesting information about *their* fate; they should not be taken to indicate the metabolism of phospholipid fatty acids in general. The points made in the paragraph on the significance of elaidic acid as labeling agent apply equally well to fatty acids labeled by conjugation.

Conjugated fatty acids when administered *per os* are incorporated into both phospholipid and neutral-fat fractions of the intestinal mucosa (89). Interestingly enough, the entrance of these fatty acids occurred more rapidly into

neutral fat than into phospholipid. Maximum incorporation into neutral fat of the mucosa took place at one hour, at which time over 50 per cent of the triglyceride fatty acids were conjugated fatty acids. About 6 per cent of phospholipid fatty acids was replaced by tagged fatty acids at one hour, and a maximum of about 15 per cent was found at 8 hours. Barnes *et al.* (89) urge caution in accepting the view that phosphorylation of fat is an essential step in fat transport.

Barnes *et al.* (40, 41, 42) studied the effects of adrenalectomy upon the absorption of conjugated fatty acids and their incorporation into phospholipid and neutral-fat fractions of intestinal mucosa and liver. The rate of entrance of tagged fatty acids into phospholipid of intestinal mucosa and liver is not impaired in the adrenalectomized rat maintained in good condition by salt therapy. This reaction apparently does not require the adrenal cortical hormone. In the absence of the adrenal glands, the incorporation of tagged fatty acids into neutral fat is markedly decreased in the liver but proceeds at a normal rate in the intestinal mucosa; the administration of cortin restores the normal rate of entrance of conjugated fatty acids into liver triglycerides.

Barnes *et al.* (90) observed a decrease in the incorporation of labeled fatty acids into the phospholipids of the intestinal mucosa of fat-deficient rats. The incorporation of conjugated fatty acids into intestinal mucosa phospholipids *in vitro* was demonstrated (91).

3. *Deuterated fatty acids.* Cavanagh and Raper (92) fed a labeled fat prepared by the partial deuteration of unboiled linseed oil. Four and eighty-seven hundredths per cent of the H atoms of the fat was deuterium. This fat was fed to rats and the atoms per cent deuterium incorporated into acetone-insoluble lipids (phospholipids) and into glycerides compared in various tissues. The incorporation of fatty acids containing D into phospholipid was shown for liver, kidney, brain, blood corpuscles and plasma. At all intervals the highest atoms per cent D was observed in liver phospholipids. The atoms per cent D found in kidney phospholipid was approximately one-third of that found in liver phospholipid. The lowest incorporation of D was observed in brain phospholipid. Since plasma phospholipid contained a lower atoms per cent D than liver at the end of 6 hours, these workers suggest that the incorporation of deuterated fatty acids into phospholipid occurred in the liver. The deposition of deuterated fatty acids into the phospholipid and neutral-fat fractions of liver and depot fat has also been noted by Barrett, Best and Ridout (93).

Sperry, Waelsch and Stoyanoff (94) also fed a deuterated linseed oil to rats and showed that only *traces* of labeled fatty acids made their appearance in the brain, while the largest concentrations appeared in liver and small intestine. From experiments in which growing rats were fed D₂O, Sperry *et al.* (95, 96) have concluded that the brain synthesizes a large part or even all of its own fatty acids. This evidence, along with that recorded above in the section dealing with the phosphate radical, indicates that the incorporation of both fatty acids and phosphate into phospholipid molecules of the brain takes place to a considerable extent within the brain itself.

III. LABELING OF THE NITROGENOUS BASE. Stetten (28, 29) has followed the fate of dietary choline and ethanolamine by means of heavy nitrogen (N^{15}). Within 3 days, at least 21 per cent of the choline present as phospholipid in the whole animal was replaced by isotopic dietary choline. Within the same period, at least 28 per cent of the phospholipid ethanolamine of the animal body was replaced by the labeled ethanolamine fed. These values show that a rapid regeneration of the phospholipid molecule occurs with respect to its nitrogenous base. The incorporation of labeled dietary choline into phospholipid was found to be most active in the liver, less active in the gastrointestinal tract, and least active in the brain.

Stetten has shown that ethanolamine is rapidly converted to choline in the animal body. The extent of this conversion was shown in an experiment in which, over an interval of 3 days, dietary ethanolamine contributed at least 11.5 per cent of phospholipid choline. The methylation of ethanolamine to choline was observed even in rats maintained on diets poor enough in methyl groups to produce fatty livers (29). Interestingly enough, the demethylation of choline to ethanolamine was not observed.

Welch (26, 27) has demonstrated the incorporation of arsenocholine (in which arsenic replaces the nitrogen of choline) into liver and brain lecithin.

The writer is indebted to A. Taurog, H. Schachner, D. Zilversmit and M. Morton for assistance and suggestions in the preparation of this manuscript.

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THE FUNCTIONAL REPAIR OF NERVOUS TISSUE

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Repair of nervous tissue comprises all the processes by which, after severance or other lesion, the capacity for effective function is restored. The power of these restorative processes is such that in Amphibia full functional capacity may return even when whole new stretches of nerve have to be made, or after severance of the spinal cord. In Mammals, where the functions served by the nervous system are more complex, full return of function is more rare. In the peripheral nerves it occurs only after simple lesions such as a crush, and only limited powers of regeneration remain in the central nervous system, though they may be sufficient to produce functional union between the severed ends of the spinal cord (Sugar and Gerard, 1941).

It is necessary to emphasise the truism that regeneration only ends with recovery because research on the subject has not, in the past, clarified equally all of the stages which must take place before the whole process is complete. The great controversy whether autogenous regeneration can occur in an isolated peripheral stump forced some of the best workers to emphasise especially the growth of the axon tip, until, in the minds of many, that process alone has become synonymous with nervous regeneration as whole. But the journey of the axon tip to an end organ is only the most dramatic of the phases in the process of regeneration, and its arrival is alone no guarantee of the return of useful function. Before that result is achieved a whole series of processes must occur; they may be divided as follows:

1. Closure of the gap between the severed stumps, mainly by the outgrowth of Schwann cells from the peripheral stump.
2. Retrograde degeneration of the cut central ends of the nerve fibres, and the sending out of many fine branches.
3. The progress of the tips of the axons from the central stump across the scar to the peripheral stump.
4. Break-up of the axons and myelin in the peripheral stump, and removal of their remains by macrophages.
5. Multiplication of the nuclei of the Schwann cells and increase in the volume of their cytoplasm to make the Schwann-bands (bands of v. Büngner¹) which eventually fill the old sheaths.
6. Progress of the axon tips along the peripheral stump, spinning out new fibres behind them.
7. The arrival of the growing tips at an end organ and the making of an union with it. This union may at first be atypical, and we should include here the subsequent process of normalisation.
8. The increase in diameter of the fibres originally laid down, their medulla-

¹ Boeke (1935) points out that Hanken described these bands in a doctor's thesis of 1885.

tion, and any other processes which may be necessary before they can conduct such impulses as can produce effective function. These processes of adjustment may possibly include not only changes in the nerve fibres, their cells of origin and perhaps their central connections as a result of the new peripheral connections, but also the addition of subsequent fibres, led along into the periphery guided by contact with the successful fibres (see Weiss 1941a).

For a satisfactory understanding of regeneration we need information about the way in which each of these processes occur, and especially about their rates. Only with such knowledge shall we be able to assess the influence on the final result of incompleteness or delay at any stage. But in spite of the great amount of work on the subject the processes listed under several of the above headings are known only in outline, and for none of them have we that detailed quantitative information which is necessary for practical purposes. As in other biological fields the fact that the stages successively reached during regeneration can be made the subject of fascinating visual examination by the microscope has to some extent hindered study of the processes by which these stages are reached. Indeed the routine methods of the neurohistologist are for the most part quite unsuited for making continuous observations. The considerable success obtained by Williams (1930), Speidel (1932, etc.) and Clark, Clark and Williams (1934) by simple continuous observation of the unstained nerves shows how much can be achieved in this way.

In this review an attempt will be made to summarise recent advances in knowledge about each of the stages into which nervous regeneration can be divided, with special attention to the way in which each process occurs and to its rate of progress. In this way we may achieve an understanding of the factors which determine the time necessary for recovery, and also an insight into the obstacles which are most likely to prevent recovery or reduce its effectiveness. It is especially necessary to make such a review because of advances during the past decade in our knowledge of nervous functioning, and particularly of the importance of the presence of fibres of various sizes (see Grundfest, 1940; Erlanger and Gasser, 1937). There have also been great advances recently in understanding of the detailed structure of nerve, and the significance of this structure for functioning (see Schmitt and Bear, 1939). As there are already several excellent reviews of earlier work on nervous regeneration² the ground covered by them will, so far as possible, be avoided.

The composition of normal nerves. In Mammals effective function never results from the conduction of a single nerve impulse, but depends on series of impulses in each fibre, properly synchronised with those in many other fibres. We may consider each single fibre as a unit only if we remember that it plays a part in biologically valuable functioning only when acting in proper combination with others. If the process of regeneration is to be effective it must restore the fibres to a state in which this again becomes possible. The whole secret of a proper understanding of the more subtle variables of nervous regeneration is

² Stroebe (1895), Perroncito (1907), Boeke (1921 and 1935), Nageotte (1932), Spielmeyer (1929), Cajal (1928), Lee (1929), Huber (1922) and Rossi and Gastaldi (1935).

an appreciation of the status of each individual fibre as a propagator of impulses of appropriate velocity and as a member of a company, with which its work must harmonise. Since we do not yet fully understand this status even in a normal nerve it is not surprising that we are unable to explain the tantalising delay and discouraging imperfections which may occur during regeneration in man. The information we possess about facilitation both at central synapses and neuromyal junctions makes it seem probable, however, that the first requisite for proper functioning of a fibre is that it must carry impulses at the right speed and frequency, and we have now reason to suppose that this implies that it must have the right diameter and degree of medullation. Since our problem is to discover how nerves return to their normal state we must first enquire how the various components of a nerve fibre differ in the differing fibre types which have been recognised. The normal nerve fibre is a system able to propagate messages by virtue of its composition of concentric layers of substance. At the centre is the axoplasm, a complex mixture, probably of semi-fluid consistency and containing at least some long submicroscopic rodlets arranged parallel to the main axis of the fibre. This longitudinal organisation is shown by the birefringence of the axon (Bear, Schmitt and Young, 1937), and perhaps by the "neurofibrils" which the axoplasm sometimes presents. There is no evidence to show how, if at all, this longitudinal organisation is related to functioning, and indeed Schmitt and Schmitt (1940) find that there is no change in birefringence during the passage of a nerve impulse. In stained preparations there appear to be some differences in the composition of the axoplasm in different animals, but there is no evidence that there are significant differences between the axoplasm of fibres of different types in any one animal. The diameter of the axon, however, has an important influence on function, impulses moving faster in the larger axons (see Pumphrey and Young, 1938). Many other features, such as the refractory period, also vary with diameter, but the influence of variation in diameter alone, apart from medullation, has been too little investigated.

Bounding the axoplasm is presumed to be a membrane, the axolemma. This has never been convincingly demonstrated under the microscope, though Boveri and others have described an inner neurilemma which, as Mönckeberg and Bethe (1899) remark, may be the same thing as the 'Axencylinderscheide'. As the latter authors observe, this inner membrane is quite different from the neurilemma and should not be given a name which suggests any similarity.

However, there is certainly a layer which has very peculiar properties, recently summarised by Cole (1941) and including a capacity of $1.1 \mu\text{F}/\text{cm}^2$, a resistance of a few hundred ohms/ cm^2 , an element of inductance comparable to $0.2 \text{ henry}/\text{cm}^2$, and a marked rectifying action. With such properties it is not surprising to find that the membrane is differentially permeable to ions and that there is a very high concentration of K^+ and low of Na^+ and Cl^- within the fibre (Bear and Schmitt, 1939; Webb and Young, 1940). There is every reason to suppose that these properties of the membrane are essential features of the mechanism by which impulses are propagated, but at present we know little of how they vary in different fibres.

Around the axon there is often a visible myelin sheath, which may be described as "a type of smectic structure in which concentric sheets of protein are interspersed between layers of lipoids so as to form structures which are repeated periodically in a radial direction" (Schmitt and Bear, 1939, p. 38). This layer is interrupted at intervals by the nodes of Ranvier and perhaps the incisures of Schmidt-Lanterman. The distance between the nodes increases with diameter (Hursh, 1939) and is probably an important characteristic of the functioning of the fibre. Even those fibres which are usually considered non-medullated possess a layer of orientated fatty molecules around the axon, this being revealed by special methods, even when it contains too little fat to appear after treatment with osmium tetroxide. It therefore seems probable that some such arrangement is essential for all nervous conduction. The thickness of the myelin sheath must affect many of the fundamental properties of nerve, but there are as yet few controlled data in which medullation is considered apart from diameter. Certainly the presence of a thick myelin sheath increases the speed of conduction of impulses. For instance prawns have heavily medullated fibres, and these conduct much faster than any other crustacean fibres of similar diameter (Holmes, Pumphrey and Young, 1941). In vertebrates the A fibres, which have the greatest conduction velocity, are heavily medullated, but as they are also of greater diameter than other fibres it has not yet been possible to say exactly how the two factors are related. The important point is that proper functioning presumably depends on the existence of fibres of various sizes, degrees of medullation and hence of conduction velocity. We have still however only fragmentary information about the rates of conduction of the impulses set up by various afferent stimuli (see Zotterman, 1939; Erlanger and Gasser, 1937). To be effective regeneration should presumably restore these detailed features, but we have as yet no accurate information as to the degree to which this can be accomplished (see p. 349). We do not even know how the diameters and medullation are normally produced; do they depend on the size of the cell body from which the fibre issues, or on some property residing in the environment of the peripheral fibres themselves? Hursh (1939) has shown that during post-natal growth in the cat diameter and conduction velocity increase in proportion to the length of the leg, so that the conduction time remains constant as the animal grows.

The outer boundary of the myelin is usually said to be marked by the neurilemma or sheath of Schwann, but visual knowledge about this region is not satisfactory, and there is much obscurity about the terminology (see Munzer, 1939). The term neurilemma was used before the time of Schwann to mean the connective tissue sheaths of the nerve, including what (following Key and Retzius, 1873) we now know as epi-, peri- and endo-neurium. This use still persists, for instance Cajal sometimes used neurilemma for epineurium (e.g., 1933, p. 310, though on p. 313 he uses it in quite a different sense). In 1839 Schwann described "a structureless and peculiar membrane, of finely granulated appearance", or "thin pale membrano" which covers the "white substance" (myelin) and has "hitherto been included with the neurilemma". He emphasised that

this membrane was not fibrous but granular and "a cell nucleus is here and there seen lying in the pale border which surrounds the white substance". These phrases clearly establish that Schwann supposed that his pale membrane was a cell, and that it was distinct from the "neurilemma".

Elsewhere he says that the "nucleus seems . . . to lie upon the inner surface of the cell membrane", and this is the relationship which has been accepted by most later workers. For instance Cajal (1928, p. 42) says "nerve fibres . . . show, under the membrane of Schwann, and outside the myelin, a very thin clear zone, finely granular . . . (this zone) shows an ovoid nucleus" and is in fact the cell of Schwann. The most general belief is that this protoplasmic layer, the cell of Schwann, more or less completely surrounds the myelin, but its exact extent has never been demonstrated. Nemiloff (1908), Cajal (1928), Nageotte (1932), Boeke (1935) and others have described it as a thin layer or set of strands over the whole myelin surface with slight thickenings near the nodes, and Nemiloff and others have also supposed it to extend within the myelin. It is admitted, however, that the layer is so thin that it often cannot be seen except near the nucleus (v. Büngner, 1891).

The "membrane of Schwann" on the other hand can be clearly seen in teased preparations and sections as a thin layer, closely adherent to the myelin, and curving in at the nodes of Ranvier. The relation of this membrane to the surrounding connective tissues has, however, also given rise to controversy. According to the account of Plenk (1934) there is no special relationship of the layer usually called the membrane of Schwann to the cell of Schwann. Following this view the sheaths are best described by speaking of an inner endoneurium, or sheath of Plenk and Laidlaw (1930), consisting of a network of argyrophil reticulin fibres, and an outer endoneurium of collagenous longitudinal fibres (sheath of Key and Retzius, 1873). Cajal indeed recognises that what he calls the "membrane of Schwann" is not distinct from the endoneurium, and he speaks of the collagenous tube as "not a new peritubular sheath, but . . . a re-enforcement of the membrane of Schwann" (1928, p. 63), or "a fibrillar reinforcing cover (sheath of Retzius) around the membrane of Schwann" (1933, p. 320).

The problem cannot be satisfactorily solved until we know more about the chemical nature and histogenesis of these membranes. Plenk's solution has an attractive simplicity, and it must be admitted that there is no reason, except their close proximity, for supposing that the membrane of Schwann is a product of the cell of that name. On the other hand there is also no more reason to assume it to be the product of fibroblasts, and it certainly stains somewhat differently from collagen.

The term "neurilemma" has thus become hopelessly confused, indeed Key and Retzius suggested as early as 1873 that it should be abandoned. However it has become so widely used that there is perhaps no harm in retaining both it and the term sheath or membrane of Schwann for the same structure, namely, the closely adherent sheath (inner endoneurium) which bends in at the nodes. This is not the sense in which Schwann used "neurilemma", but that cannot be helped

if we are to retain the modern use. The terms neurilemma, sheath or membrane of Schwann will not be taken here to include the protoplasmic component, which will be referred to as the cell of Schwann. It is most important to make this distinction since during degeneration the protoplasm of the cell behaves very differently from that membrane which we are now agreeing to call the neurilemma or sheath of Schwann.

A nerve fibre, then, is a unit made up of these various substances, and able to carry nerve impulses. Each fibre is able to play its particular part in normal functioning by virtue of *a*, its central and peripheral connections, and *b*, the speed and the frequency with which it conducts impulses. Nerve fibres are not all alike but differ especially in diameter, length of internode and thickness of medullation, these being the factors which control speed and maximum frequency of conduction. The study of regeneration is therefore the study of those processes by which *a*, the appropriate connections, and *b*, the appropriate diameters and degrees of medullation are restored.

In either normal development or regeneration the production of a nerve fibre is the result of the combined activity of at least three types of cell, the neuron, the Schwann cells and the fibroblasts of the endoneurium. But the substances of the fibre may be extracellular (e.g., collagen and myelin) and the whole fibre as a unit transcends the cells in function, and probably in development. On the other hand both during development and after damage the cells certainly act separately from each other. The migrations of the Schwann cells as described by Harrison or Speidel are in striking contrast with their passive state as parts of the lamellated structure of the normal nerve fibre.

After severance of any fibre the whole system breaks up in the portions no longer connected with a nerve cell body, the axoplasm and myelin disappearing. But the nerve as a whole does not disappear, even if it is left uninnervated for long periods, though it shrinks to about one half of its diameter. The Schwann cells and connective tissues remain and undergo changes anticipatory of re-innervation. Indeed these tissues, and especially the endoneurium, are able to maintain the pattern of the nerve, forming a system of "Schwann tubes" in spite of the absence of nerve fibres. Moreover these tubes are not all equal, each able to re-form any type of nerve fibre. Besides the peripheral connections which allow each Schwann tube to lead a new fibre back only to certain districts, each also maintains, even in degeneration, a certain specificity of diameter. This means that it can reconstruct only a certain range of fibre types (p. 348). When axons ultimately return their reconstruction into functional fibres is therefore very largely influenced by the environment which each growing fibre finds in the peripheral stump. Full function will only recover if an adequate number of central fibres reaches tubes in the peripheral stump which are sufficiently appropriate to allow them to mature properly and to reach endings not very different from those with which they were formerly connected.

Comparison of regeneration with development of a nerve. The study of the mechanism by which normal nerves are produced in ontogeny has much that is relevant to the investigation of regeneration (see Detwiler, 1936; Weiss, 1941a, b).

Following Weiss we may recognise that "the establishment of a peripheral nerve involves three overlapping phases: First the *free* outgrowth of a group of pioneering fibres through non-nervous surroundings. Second, the *bound* outgrowth of subsequent fibre generations along the line laid down by the pioneering fibres. And third, the towing process in which the nerve is drawn out by 'growth' " (1941, p. 165). We have only very little information about how during development the outgrowth is organised to ensure proper connections. After rejecting various current theories of attraction of fibres from a distance (chemotropism etc., see p. 335). Weiss concludes that they are guided by "contact action", that is to say, they grow along surfaces or lines of stress which are produced by or among the developing organs with which they come into relationship. He suggests further that fibres which make successful connections "acquired some contact property which made their surface sticky, or otherwise a pathway of preferential application, for other fibres growing out subsequently" (p. 181). But as regards the problem of how the nerve fibres come to have appropriate sizes we have no more information during development than during regeneration.

For comparison with ontogeny, and as a general working hypothesis we may suggest that regeneration in adult mammals consists essentially of: 1. The free sprouting of many new nerve fibre branches from the central stump, and cords of Schwann cells from the peripheral one, so that some of the former meet the latter and are conducted along to the end-organs. 2. The maturation by increase in diameter and medullation of some fibres, perhaps especially those which make functional connections. 3. The atrophy of the rest. The schemes for regeneration and development are similar in postulating an initial outgrowth which is "free" except of course that it takes place especially easily along certain surfaces or lines of stress. However no stage similar to that of Weiss' "bound" phase of subsequent fibre growth is here postulated for regeneration, for reasons given on p. 369. On the other hand, during regeneration the fibres grow out into a field containing pathways that are already partly specifically differentiated, namely, the Schwann tubes of the peripheral stump.

If this thesis is true it means that successful restitution of function is the result of the development of those fibres whose central and peripheral connections are such, after the outgrowth, that they can be used, rather than the direction by successful fibres of others following in their wake or by changes in central connections. This is the hypothesis in the light of which we shall examine the various phases of regeneration, but it must be recognised how much is obscure, even in its formulation.

The union scar. The structural union between the sutured ends of a nerve is formed by the combined action of Schwann cells and fibroblasts. The former very rapidly undergo a striking metamorphosis into long fibrous structures which pour out from the stumps into the space between the cut ends. This remarkable process has been known for some time but has not received the attention it deserves (see Ingebrigtsen, 1916; Kirk and Lewis, 1917; Nageotte, 1932; Masson, 1932). That these cells should be able to move is perhaps not surprising in view of their migrations during development (Harrison, 1924). Speidel (1932) has

watched their wanderings during regeneration in the tail of the tadpole, and Ingebrigtsen (1916) first showed that they make similar movements in tissue culture.

It is important to realise the rapidity and extent of this outgrowth. Kirk and Lewis report that the Schwann nuclei near the scar begin to accumulate protoplasm after 24-36 hours and "after removal of a segment 12 mm long the protoplasmic bands have completely bridged the tube of serum at the 6th day". They considered that the cells grow out from both stumps, but more rapidly from the central. This may perhaps be true for some stages, but Young et al. (1940) found in the rabbit that if large gaps were left between the stumps the outgrowth from the distal end was, at least in the early stages, the more extensive. Indeed a central stump which is left isolated and very remote from a peripheral one builds a broad bulb, but not usually a long outgrowth. It seems that the Schwann cells do not continue their migratory activities when they are innervated, and indeed this agrees with the observations of Ingebrigtsen (1916), confirmed by Abercrombie and Johnson (1942), that in tissue culture they only grow out from degenerated stumps, not from fresh nerve.

On the other hand the proliferation from the peripheral stump is usually very apparent as a whitish cloud or set of strands, the form and length depending on the surrounding conditions. Examination of sections shows parallel rows of Schwann cells running through the fibrous tissue. Mitotic divisions can be seen, but it is not clear whether they occur chiefly towards the tip or throughout the tissue which has wandered out. Nor is it yet certain whether the outgrowth continues only for the period during which there is nuclear proliferation in the peripheral stump or indefinitely. If the latter then very large outgrowths should be found after a peripheral stump has been left isolated for a long time. In our series of rabbit's nerves we have not found this to be the case. Quite long outgrowths may be rapidly formed during the early stages, but they do not increase continually.

There are few data to show what factors, if any, make for attraction of these outgrowths toward the opposite (central) stump. They tend to grow along any supporting line which they can obtain. Thus in some experiments a portion was excised from the tibial nerve in the thigh, while the peroneal, which lies alongside it, was left intact. The outgrowth from the peripheral stump then took place very readily alongside the intact nerve, in some cases producing thick and definite strands which we christened "ghost nerves" (fig. 1).

The observations of Kirk and Lewis do not allow separate calculation of the rate of outgrowth from the two stumps, but measuring the extent of the proliferation in cases in rabbits in which no union had been effected across gaps we have found rates of outgrowth from the peripheral stump approaching 1 mm/day, assuming no latent period before outgrowth begins. Speidel (1932) saw movements of Schwann cells by as much as 200μ per 24 hours in newly regenerated zones in the tails of tadpoles. In our series the amount of outgrowth varies considerably, for reasons which are not wholly clear. The most vigorous outgrowths were obtained not from freshly cut stumps, but from those which had been previously de-

generated for some time. This agrees with the finding by Abercrombie and Johnson (1942) that the power of emigration of Schwann cells *in vitro* reaches its maximum after about 20 days of degeneration.

This power of outgrowth of Schwann cells must be of the greatest importance for the regeneration of nerves, since it is the main agent by which physical continuity is restored between the intact axons of the central stump and the old pathways in the peripheral one. In some animals considerable gaps can certainly be bridged in this way. Thus Gutmann and Sanders (1942) have found partial recovery of the power of spreading the toes in the rabbit 117 days after complete excision of 2 cm. from the peroneal nerve which mediates this function, a thick strand having joined the stumps. After simple severance and suture at this level recovery occurs after about 80 days. However, in addition to the one animal which showed functional recovery after removal of a stretch of nerve five others were operated but failed to recover within 180 days. The restoration of function by bridging gaps in this way is therefore both uncertain and incomplete. Vanlair (1894) found that after removal of 1 to 3 cm. of tibial nerve

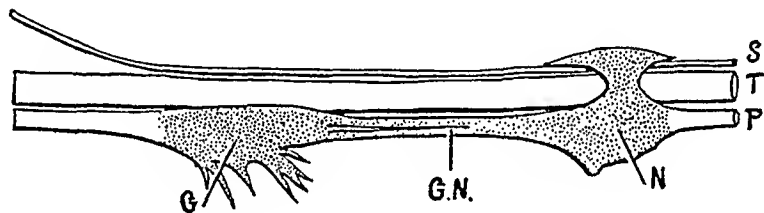


Fig. 1. Sciatic nerve of the rabbit in the thigh 180 days after removal of a stretch of 2 cm. from the peroneal division, *P*. There is a neuroma, *N*, on the central and a glioma, *G*, on the peripheral stump. Between these there is a "ghost nerve" *G.N.* The approximate boundaries of the new tissue formed are shown by the stippling. *T*, tibial division; *S*, sural division.

from dogs functional recovery could occur (after long delay), but not after removal of 4 cm.

It is probable that gaps of the extent of 1 to 3 mm. can be effectively crossed in this way in Man. But for return of the more complex functions nothing but the best conducting pathway for new fibres is adequate, and it is doubtful whether the outgrowing Schwann cells can provide this over long distances. It is important in any case to realise that the body is provided not merely with the power to make new connections when the old are cut, but actually to build new stretches of nerve. Under natural conditions such provision is indeed a necessary antecedent to the whole process of regeneration, there being no other surgeon available to join the stumps together. It would perhaps be worth considering the possibility of devising means of encouraging and directing this process so as to make it possible to provide an effective bridge across large gaps. Probably such new stretches of nerve play a part in the recovery of not a small proportion of human cases, even with existing technique.

Besides the Schwann cells there are also abundant fibroblasts in the region between the two stumps, and their products no doubt produce most, if not all of

the tensile strength of the junction. Presumably the two tissues exert powerful influences on each other, but it is not clear which, if either, of them controls orientation in the newly formed tissue. One main difficulty for the histologist is to distinguish between the two types of cells at all. The only really safe guide is the characteristic method of growth of the Schwann cells in strands, several side by side, which produces an appearance different from that of fibroblasts, even when the latter are longitudinally orientated.

Retrograde degeneration and sprouting from the central stump. The changes which go on in the central stump have been described by many workers (see especially Perroneito, 1907; Bersou, 1912; Ranson, 1912; Cajal, 1928). Essentially they consist in the destruction of the region immediately adjacent to the cut, and

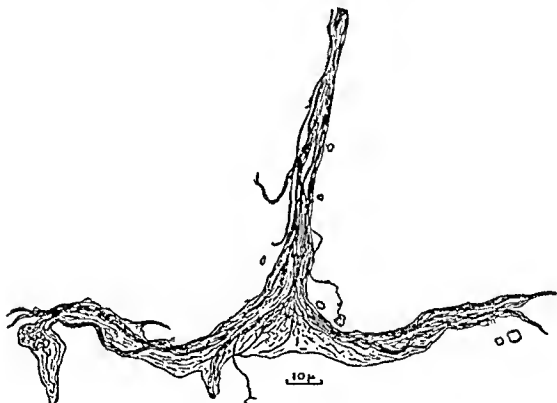


Fig. 2. Outgrowth from the central end of a human axon divided 26 days previously: The axoplasm has flowed out into strands, mainly laterally. Bodian's fixation and staining

the giving out by the stretch of nerve above this of very numerous branches. The retrograde degeneration includes a break-up of the myelin as well as the axon, and there is considerable proliferation of the nuclei of the Schwann cells. These changes begin about the 3rd day and may proceed for a considerable distance (up to 2 cm., Ranson, 1912, 3-4 cm., Mönckeberg and Bethe, 1899). It is often forgotten that there may also be a very considerable complete central degeneration of many fibres and their cells after severance of a nerve. Thus Ranson (1906) found that half of the cells in a spinal ganglion disappear within two months when the nerve is cut close to the ganglion in rats. The cells connected with non-medullated fibres are particularly vulnerable (Ranson). Greenman (see p. 335) has found that fibres may even degenerate on the opposite side of the body.

Since the work of Nissl it has been known that the cell bodies of neurons whose axons have been severed may show the changes known as chromatolysis (see Bielschowsky, 1935). This begins as early as the first day, the Nissl granules disappearing from the dendrites and then later from the cell body. In extreme stages the whole cell becomes pale and its nucleus excentric in position. But the degree of chromatolysis is very variable. It is more severe when the lesion is close to the cell body, and especially if the nerve is evulsed rather than merely cut. Romanes (1941) has shown that severance of nerves in the distal part of the limb may fail to produce any chromatolysis. Possibly the changes are concerned with the disturbance of the arrangement of the axoplasm which results from the outflow from the cut point (see below) which must upset the structural and ultrastructural arrangement of the substances at least in the neighbourhood, if not also further away. The maximum extent of chromatolysis is seen during the second and third weeks, after which the Nissl substance begins to return, first around the nucleus and eventually throughout the cell.

The appearance of the numerous new sprouts has been many times described, but it is still not clear by what process they are produced. Since the substance of some axons at least is semi-fluid and flows freely from the cut end of a large nerve fibre (Young, 1935, 1936), it is possible that the budding consists essentially of such an outpouring. Gutmann and Sanders (unpublished) find that the axons in the central stump above a suture are always smaller than normal, as if they had become depleted by an outflow. On the other hand the brush-like appearance which is so often produced strongly suggests the division of the original axon into a number of fibrillae. As Cajal acutely puts it "(The) phenomena of neurofibrillar creation oblige one to believe that within the ultramicroscopic units in the protoplasmic skeleton there are present transversal and longitudinal divisions, which cause the formation of new series or linear colonies" (1928, p. 162).

In any case this process is certainly of crucial importance for the whole act of regeneration, since it is only by virtue of the presence of very large numbers of tentative outgrowths that appropriate connections are ultimately restored. It is difficult to give an accurate estimate of the number of branches so formed. Perroncito's figure 27 shows 24 of them coming from one fibre, but very many of those shown are thick and clearly likely to divide again. Ranson (1912) estimates "fifty or even more", so perhaps 100 branches is not an over-estimate. It would be interesting to know whether large fibres give more branches than small ones and thus obtain an advantage in making of new connections. Castro (1930) saw very many on regenerating postganglionic fibres. Levi (1941) reports that in tissue culture the number of branches produced by a fibre is increased by irradiation with radium. Greenman (1913) has investigated the extent of the increase in number of medullated fibres at various levels central to the lesion.

The new buds are formed as early as the third hour after section, according to Perroncito, but it may be that these early buds are abortive and disappear. Certainly the greater number of new sprouts are formed after retrograde degeneration has taken place. Various formations are seen, the advancing tips appearing as brushes, bulbs and especially rings, which are present in very great numbers

during the first three weeks. We have seen them in severed human nerves as early as the 4th and as late as 26th day, but it may be that they occur outside these limits. It is only necessary to look at the beautiful figures of Cajal and Perroncito to see that the processes during these early stages are not simple and should not be thought of too diagrammatically. Branches are put out in all directions, and often in most unexpected ways. Thus Perroncito notes that several fibres may join at a single ring, or a fibre may proceed from one ring to another. Silver preparations at this stage often show isolated rings attached to no fibres (fig. 2) and these are probably not artefacts but points which, failing to advance, are degenerating. The fibres do not all grow down the nerve, many pass up it and, at the edges, laterally. When an outgrowing tip becomes obstructed there develop either large "axon bulbs" or, by a process of spiral backward growth, the very complicated spirals of Perroncito. Both of these formations are definitely the result of obstruction to the path of forward growth, and they make no effective contribution to the end-result. Dustin (1917) looked for them carefully in a series of human amputation neuromas and found that they gradually decrease with time, and are not present in stumps more than 90 days after amputation. In our series of rabbit neuromas we have also noticed this decline, though some axonic bulbs may remain for very long periods. The absorption of these conspicuous but useless structures gives a clear demonstration that some process of adjustment is continually going on in a nerve, with removal of superfluous axons or branches. Similarly there is evidence that branches growing out from the central stump which do not reach the peripheral one are sometimes, though not always, absorbed, and the small loops, so abundant at the beginning of regeneration, are not found after about three weeks. Unfortunately we know nothing of the nature of the "trophic" influences which protect functioning fibres from such destruction.

Neuromas and methods for preventing their formation. The neuroma which forms on the central end of a severed nerve is a result of the power of outgrowth, exercised without the collaboration of the Schwann tubes of a peripheral stump. Guttmann and Medawar (1942) have proved that it is the "pressure" of forward growth of nerve fibres which produces a bulb, by tying two ligatures on a nerve. A bulb then forms only above the upper one and not at the lower, at which, of course, there are no nerve fibres. Nerve fibres accompanied by central Schwann cells may progress for some distance forwards through foreign tissues, but usually they grow not straight but in all directions producing a large bulb, rather than a long outgrowth. Dustin (1917), Huber and Lewis (1920) and Marinesco (1918) have analysed the histology of these bulbs and the first named recognises in them three regions. *a*, The fascicular zone in which the bundles have undergone retrograde degeneration and are separated from each other by an "interstitial oedema"; *b*, the plexiform zone where the bundles spread out, changing direction abruptly at the level of the cut, and *c*, the trabecular zone, consisting of outgrowth into neighbouring tissues. The bulb is thus made up of nerve fibres and proliferated Schwann cells which have pushed out from the cut end, usually as isolated strands, running in irregular directions, each containing one or

few axons and surrounded by a collagenous sheath. Throughout the whole swollen region there is a great deal of fibrous tissue.

Such end bulbs go on growing slowly for a long time, perhaps indefinitely. Both in human cases and animal experiments the size of bulb is found to increase with the time after severance, though the rate of growth is low. It is not known what conditions produce bulbs which give pain (see Corner, 1918, 1919). In a proportion of cases it is infection, and in others simple mechanical irritation. The size of the bulb is often an important factor, though very small bulbs may be painful if they form in exposed places.

The ideal method for preventing the formation of neuromas might be discovered if we understood why it is that outgrowth stops when a nerve fibre reaches its end organ. Alternatively it might be possible to encourage that retrograde degeneration and atrophy of cell bodies which certainly takes place in some cells when their axons are severed (Ranson, 1906). Until research has yielded further information about these problems the only methods available for inhibition depend on killing the fibres as far back as possible by making injections into the nerve, and then placing obstacles in the way of regeneration by suturing the epineurium (see Stookey, 1922). The injection of alcohol has been used at least since the time of Weir Mitchell. However Bersou (1912) and Huber (1920) showed that alcohol injection does not indefinitely prevent regeneration, and Guttmann and Medawar (1942) have recently re-investigated the question, injecting various substances into cut rabbit nerves. They find that a region injected with alcohol is rapidly invaded by macrophages, among which new fibres grow out readily, so that no inhibition is produced and a large neuroma is formed. On the other hand tissue injected with strong formol is removed only very slowly and the resultant tissue provides a very poor path for new fibres. Even after a year such nerves present no end bulb, but taper down to a fine strand. Formol has already been used by Foerster and others in man. Satisfactory "inhibition" was also produced by Guttmann and Medawar by injections of solutions of gentian violet, a very powerful protoplasmic poison, the nerve appearing as a tapering strand even at long periods after severance, when controls had produced large bulbs.

Crossing of the scar. The period of re-organisation of the central stump and initiation of outgrowth lasts only for a few days. Cajal (1928) estimates that by the end of the 2nd day fibres begin to enter the scar proper, and Cajal, Perroncito and Ranson all agree that some fibres are found beyond the central stump on the second day, though they may be abortive outgrowths, and the majority certainly come much later. There has been considerable controversy as to how the fibres are supported during their passage across to the peripheral stump. Following the experiments of Harrison (1910) which showed that in tissue culture nerve fibres can grow out freely without other tissues, some workers have supposed that during regeneration they also grow freely at their tips. Others have maintained that from the outset they are in more or less intimate connection with the Schwann cells in the scar. Nageotte (1932) has especially developed this view, holding that nervous tissue is always supported by ectodermal Schwann

tissue and never comes into contact with mesodermal derivatives. Lewis and Kirk (1916) state definitely that "the bands grow down first, and the nerve fibres follow along them", and that the bands are "nature's effort for ensuring a pathway for the regenerating axis cylinders". Among those of this school opinions differ as to whether the axons lie on or within the bands. Lewis and Kirk say that "sometimes the fibre is embedded in the cytoplasm of the protoplasmic band, sometimes it lies very near the surface".

On the other hand Cajal (1928) maintained that axon tips could definitely grow out freely without special cellular support, and the observations of Speidel (1933) seem to support this view; "the early sprouts grow into mesenchymatous spaces, encountering a loose network of fibroblasts (p. 60)". He saw no sign that the new nerve sprouts are orientated by the Schwann cells, which indeed become applied to them only later (1932, p. 310).

Probably there is a certain truth in both views. During the early stages, such as those figured by Perroncito (see fig. 2), there is no question of the brushes, bulbs and rings being anything but free outgrowths. They are very small and far too numerous for each to find support. On the other hand it is very rare to find even the thinnest fibre running unsupported across the scar. In the early stages they can be seen to be attached to the surface of the Schwann cells. There can be little doubt that it is only when provided with some such support that they succeed in progressing for considerable distances. Good evidence of this is the fact that isolated central stumps are only able to make very short outgrowths, although when Schwann bands, provided by the peripheral stump are present, large gaps can be crossed (see p. 325). On the other hand once the new fibre has entered one of the old tubes of the peripheral stump it proceeds down its inner wall without, at first, necessarily making contact with a Schwann cell (p. 325). It is evident that the Schwann cell surface, though frequently used, is not the only medium over which nerve fibres can grow rapidly.

Perhaps the solution of the problem lies in considering not only the movements of the axon tip but also the conditions under which it is able to spin a fibre behind it. In spite of all the information available about the visible phenomena we know practically nothing about the process of growth as such. Should it be considered as a mere flowing, or is active synthesis of fresh axoplasm taking place, and if so where? It is not unlikely that it is only when travelling along a suitably fibrous surface that the molecules of the material behind the growing tip become so orientated as to produce a fibre. Direct evidence about this is provided by one of our rabbits in which a piece had been excised from the sciatic and the central stump injected with formaldehyde. In the resulting nerve bizarre outgrowths were found in the form of flattened leaf-like sheets, and all of these were unaccompanied by sheath cells. Indeed, as figure 2 shows, the earliest outgrowths are often of irregular form and "protean" rather than fibrous. It is only when the outgrowing matter becomes applied to a suitable surface that it advances and spins a fibre.

Factors making for good union of stumps. Under optimal conditions remarkably direct pathways are established between the two stumps even as early as

15 days after suture. More or less parallel strands run across the region of union, so that a tissue very similar to normal nerve is rapidly re-established, consisting of Schwann cells with axons attached and surrounded by collagenous sheaths. Even under the best conditions there is some criss-crossing, and of course there is no guarantee that the new fibres enter pathways similar to those with which they were previously connected. Anything which disturbs direct re-connection is to be avoided. The ideal is presumably that the stumps should present cross sections having a structure approaching that of normal nerve, and with no fibrosis of the endoneurium such as is often found in regions damaged by gun shot wounds. Unfortunately, it is not known whether good recovery can be made after sutures in which the central stump has undergone degeneration and re-innervation as a result of the injury, and contains few, or small medullated fibres. It is hoped to decide this question by careful recording of a series of cases now under examination.

While it is undesirable for the stumps to be strongly pressed against each other, close apposition is certainly the ideal. In all of our cases where there was separation, even of a small amount, the fibres were seen to run criss-cross, and the suggestion of Nageotte (1918) that such gaps should be left intentionally is dangerous. Figure 2 shows unless the new outgrowth finds suitable tissue with longitudinal orientation into which to grow it will put out irregular and lateral processes. It is the resulting plexus formation, and the consequent failure of many of the new fibres to reach the peripheral stump which makes the functional result so much less good after severance and suture of a nerve than after interruption by crushing or other means which conserve the longitudinal organisation. We do not yet know of any means by which the formation of a longitudinally organised tissue at the suture line can be encouraged. Research directed to the problem might do very much towards improving the results of nerve suture. In the meanwhile we can only emphasise the need to use every care to prevent transverse organisation or blocking of the outgrowth; sutures which pass through the substance of the nerve should of course be avoided. If gaping at the edges is allowed direct union is seen only at the centre of the nerve and care should be taken to make thorough epineurial sutures, holding, as far as possible, two flat nerve surfaces together.

Sargent and Greenfield (1919) and Guttmann (1942) have investigated the reactions set up in nerve by various types of suture material. Catgut should be avoided, since it sets up a reaction which may be sufficient to obstruct the new outgrowth. Coloured silks (especially green, but also to some extent black) were also found to set up reactions. Fine white silk or linen thread provides the most suitable of the usual materials, but Guttmann also recommends the use of fine woman's hair, which sets up very little reaction, is very small and yet easy to use.

Young and Medawar (1940) have suggested the use of concentrated fibrinogen, poured over the stumps while they are held together, and allowed to clot. This has been found most valuable for suturing small nerves, such as the peroneal of the rabbit which can only be joined very roughly by stitches. For experimental

purposes it is particularly useful to be able to make a series of similar unions, since stitched sutures of these small nerves are very variable. In man (Seddon and Medawar, 1942) the use of plasma suture is limited by the fact that it will not hold any considerable tension. It has been found successful however for primary sutures, for instance of the median or ulnar at the wrist, in which the cut ends can easily be brought together. It is especially useful for placing of grafts, particularly cable grafts and thin grafts such as are used in the hands (see Bunnell and Boyes, 1939).

Weiss (1941c) suggests that nerves be joined by inserting the ends into an arterial tube. If an artery of suitable diameter can be found this might serve to hold nerves under slight tension, but it must be difficult to obtain accurate apposition within the tube, and to avoid damage to the ends of the nerve stumps during the process of threading.

Grafts and other artificial bridges between stumps. No thoroughly satisfactory means has been devised for bridging the large gaps in nerves which often have to be repaired, especially after injuries received in warfare. The clinical and experimental literature is so confused, especially by the use of inadequate standards for assessment of recovery, that it is difficult to draw conclusions even about some procedures which have received quite extensive trial (see Sanders, 1942, for review of the literature). There is reliable evidence that autografts composed of thin strands of cutaneous nerve can survive, become innervated and give good recoveries in man and animals. Such thin grafts have been used in surgery chiefly in the facial nerve (see Duel, 1933) and in the nerves of the hands (Bunnell and Boyes, 1939), in which situations there is no doubt of their success. Sanders and Young (1942) have shown that new fibres grow through autografts nearly as fast as through a normal peripheral stump, and that there is no basis for the fear expressed by Davis and Cleveland (1934) of a delay at the lower junction. Further Gutmann and Sanders (1942) found that autografts 2 cm. long placed in the peroneal nerve of the rabbit produce recoveries of motor function which are nearly as quick and successful as can be produced by simple suture. New fibres do not penetrate autografts more quickly if they have been predegenerated as advocated by Ballance and Duel (1932), following a suggestion of Cajal. The chief advantage conferred by predegeneration is probably that suggested by Bentley and Hill (1936), that the nerves become firmer and hence easier to handle and to place in position. However it has recently been shown by Abercrombie and Johnson (1942) that Schwann cells reach their maximum powers of emigration during the period between 15 and 25 days after severance of a nerve (see p. 364) and it is therefore possible that grafts taken during this period would make better unions than would fresh ones.

It is more difficult to form an opinion about the value of autografts in larger nerves. In some cases where unsatisfactory results have been recorded the grafts were very thin or otherwise unsuitable (Platt, 1921). For a graft to offer a cross section even approximately equal to that of one of the larger nerves of the limbs it must consist of several strands of cutaneous nerve. The insertion of such cable grafts presents technical difficulties of stitching, which may be

overcome by the use of the method of Elsberg (1919) or, very conveniently, by the use of concentrated plasma. Foerster (1929) has reported good results from a series of cases in which autografts were inserted and there seems no reason why they should not be successful if sufficient material can be obtained to make a thick cable. Seddon, Young and Holmes (1942) have examined histologically a cable graft which had been removed because of the unsatisfactory state of the peripheral stump. The grafted bundles were found to have survived very well and to be full of fibres in process of medullation.

The problem of finding sufficient material to make a good graft would be easily solved if it were shown that homografts could be used, but the data about them are strangely meagre. In man there is no single case in which a satisfactorily placed homograft can be said to have been definitely a success or failure (see Bethe, 1917). Bentley and Hill (1936) found that homografts gave good recoveries in monkeys and Gutmann and Sanders (1942) that in the rabbit recovery was only slightly slower after homografting than autografting. However Sanders and Young (1942) found that homografts are sometimes invaded by very large numbers of lymphocytes and that the rate of growth of new fibres through them is variable.

The indications from functional recoveries made in animals are such as to encourage the trial of fresh *thin* homografts in suitable cases. It would perhaps be wise to resist at first the temptation to use thick grafts since these may undergo necrosis at the centre. Storage of homografts in various ways has been suggested (Dujarier and François, 1917; Bethe, 1917; Huber, 1922) and there are indications that perhaps such storage may even improve the graft (see Sanders and Young, 1942; Gutmann and Sanders, 1942).

Various other forms of graft have been suggested but are contra-indicated either because they set up violent cell reactions (hetero-grafts) or because, being foreign bodies, they have to be removed before re-innervation takes place (alcohol-fixed grafts). Sanders and Young (1942) found that invasion of these grafts was at best exceedingly slow, and Gutmann and Sanders (1942) confirm that they give little or no recovery. Theoretically it should be possible to provide some scaffolding or line of tension along which the Schwann cells from the peripheral stump could exercise their powers of outgrowth and reach the central stump. But no satisfactory means of doing this has yet been devised.

In the absence of any well-tried means of bridging gaps in nerves surgeons have resorted to various manipulative procedures, flaps, etc. (see Sanders, 1942), much the most important of which is mobilisation followed by suture with the limb suitably flexed. This is indeed at present the operation of choice for bridging a gap in a nerve. By freeing the nerve for a long length and shortening its path considerable gains can be made, and the ends of the nerve brought together while the limb is held flexed in a suitable position. After leaving an interval for union at the suture-line, the joint is then stretched (sometimes freely by the patient, sometimes by means of a turnbuckle) the nerve presumably increasing in length to the necessary extent. Success is undoubtedly frequently obtained with this method, but Hightet and Sanders (1942) have found by taking

x-ray pictures of clips placed along dog's nerves treated in this way, that no permanent lengthening of a nerve is produced by stretching. When stretching was too extreme damage was produced, and there is evidence that this also occurs in man (see Bethé, 1917; Stookey, 1922). Precautions must therefore be such as to prevent sudden or rapid stretching, which a nerve is not well suited to resist.

Mention may be made here of the blood supply of nerve, which certainly has a big influence on the functioning of its fibres (see Bülbirg and Burn, 1939). The anatomy of the blood vessels running to nerves is reviewed by Adams (1942). Many vessels enter the trunks along their length, but the supply extends for great distances, so that removal of all vessels entering over a considerable stretch of nerve may not seriously affect its function. After denervation the blood supply of the trunks remains intact, and indeed may be somewhat increased.

Neurotropism. Cajal (see 1928) first formulated the idea that new fibres are directed into the peripheral stump by some chemical attraction. This later developed into the view that the attracting stuff is produced by the "degenerating" Schwann cells, and therefore that nerves cut 10 to 15 days previously exert the strongest attraction, and should make the best grafts.

Of the many experiments designed to test this hypothesis none is decisive. Those of Forssman (1898, 1900) show that in order to reach a peripheral stump outgrowing fibres may take a direction other than straight forwards from the tip of the central stump. But he studied only late regenerates, and did not exclude that earlier fibres had wandered in all directions, only those which reached the peripheral stump becoming medullated. He claims that axons grow into brain extracts but not liver, but gives no satisfactory documentation for this claim. Weiss (1934) was unable to demonstrate any attractive influence of degenerated nerve tissue on outgrowth of nerve fibres in vitro. Tello (1914) claimed that regenerating cerebral fibres will enter a piece of peripheral nerve planted in the brain. This is a most important observation, but it would only constitute a proof of neurotropism if it could be shown that fibres from distant regions had been directed towards the graft. Sanders and Young (1942) found that fibres do not grow faster within predegenerated than fresh autografts and there is therefore no proof that a chemical attraction is produced by the degenerating Schwann cells.

Dustin (1910) reached the conclusion that there was no chemical factor, but that fibres were led along by what he calls "odogenesis" or the "route-making" action of surfaces in the scar and peripheral stump. Dustin's experiments do not constitute a decisive disproof of the chemical theory, though his view of the importance of surfaces is probably sound.

Recent experiments in tissue culture have emphasised the importance of structural factors in orientating the outgrowth, and in particular the significance of the fine structure of the medium. Ingvar (1920) claimed that axons growing in tissue culture are orientated by electrical potential gradients applied across them, but Weiss (1934) failed to find evidence of response to such influences.

Weiss showed, however, that any orientation of the substance of the plasma clot, such as may be produced by tension or local dehydration, results in a corresponding orientation of the direction of outgrowth of the axon. He produces several lines of evidence to show that the orientation of nerve fibres is produced in this way by "the mechanical structure of the ground substance." The facts described below about the way new fibres grow into the peripheral stump strongly support this view. Further he points out that it is very difficult to reconcile any theory of galvanotropism or chemotropism with the fact that neighbouring nerve fibres may grow in *both directions* along a pathway.

The theory of neurotropism is therefore not supported by any reliable evidence and many would adopt the position of Huber (in Stookey, 1922). "There is no conclusive evidence to substantiate this (chemio-attraction). . . . It has seemed to me that the purely mechanical interpretation is more nearly in accord with observed facts than any other which could be given".

The axons growing out in all directions from the central stump and Schwann cells in all directions from the peripheral stump provide the mechanism by which some fibres are re-connected with the empty tubes. From our experiments with severed nerves in rabbits we (Young, Holmes and Sanders, 1940) have seen much evidence to indicate that these two outgrowths are orientated toward each other by the tension which is exerted when stumps are cut and allowed to retract. But there is no evidence at all that growing fibres are attracted back into pathways similar to those with which they were previously connected, and indeed everything seems to indicate that the mechanism for making appropriate new connections is the provision of very many new fibres, with reliance on the accidental entrance of some into suitable channels.

Degeneration and preparation of the peripheral stump. It is, then, along the Schwann bands of the scar that the outgrowing axons travel to the peripheral stump. Since the outgrowing bands are derived as a stream from this latter they provide a conduction path straight back into the old tubes, being continuous with the bands which are formed by the Schwann cells in the peripheral stump. The changes which culminate in the formation of these bands are usually described as a degeneration, but they really include two separate sets of processes: 1, the actual degeneration, that is to say, the break-up and removal of the axons and myelin, and 2, processes of preparation for regeneration, namely, the proliferation of the Schwann cells and their conversion into protoplasmic strands.

The processes of breaking-up have been very thoroughly investigated (see Cajal, 1928; Nageotte, 1932; Weddell and Glees, 1941). The axon becomes irregular and fragments, and the myelin breaks up, first into a series of chambers, and then into the rows of round fatty granules which are so characteristic of degenerating nerve. The broken up pieces of axon and myelin are then gradually removed, mainly by the action of macrophages. Since the recognition of degeneration is of great importance for anatomical, pathological and experimental studies of nerves it would be of great value to have detailed knowledge of the time-limits of the various processes of break-up in man and animals. Changes

may begin to occur in the axons from the first moment after section (Speidel, 1935) and nearly all are swollen and beginning to fragment after 12 hours (Setterfield and Sutton, 1935; Weddell and Glees, 1941). The breaking up of the axons begins on the second day and Weddell and Glees (1941) report that all fibres show some abnormality 48 hours after section of the nerves in the ear of the rabbit. But the break-up continues for a long time. In digital nerves of man we have found it to be not wholly complete on the 26th day, at which time stretches of axon can still be distinguished, though they are of abnormal structure. In the rabbit's ear Weddell and Glees found no fibres resistant beyond the 4th day. Probably the fine fibres are more resistant than the larger ones. Thus Ranson (1912) found in the dog that whereas most of the non-medullated fibres degenerated very early some were more resistant than any medullated fibres, and survived into the third week. By the 25th day he found all axons degenerated. Experience and good judgment are therefore necessary in deciding whether fibres found in a given stretch of nerve during the 2nd and 3rd weeks after section are undegenerated or the result of regeneration. Probably most of the fibres become obviously abnormal by the 10th day, or even earlier, but the possibility of survival of short isolated stretches of axon cannot be ignored at any time during the first three weeks.

Myelin degeneration begins during the first day and proceeds rapidly, all fibres being affected to some extent within the first week. However since the first sign of degeneration is a breaking up into segments there are always considerable stretches between the breaks which, though somewhat swollen and abnormal, may simulate normal fibres at least until the end of the second week, especially when seen in cross section. New medullated fibres begin to appear in the peripheral stump during the third week (p. 347) so great care is needed in the interpretation of the presence of medullation at any time. The removal of the fatty remains takes a very long time, and there has been some controversy as to how it and the axon removal are affected. Presumably the primary change is an autolysis of the axon, deprived of those mysterious trophic influences which emanate from the cell body. Perhaps the segmentation of the myelin then follows as a mechanical result of the collapse of its contents. The chemical break-up of the myelin involves its conversion to isotropic granules of triglycerides, which begins as early as the 18th hour and is complete by the 6th day (Setterfield and Sutton, 1935).

The break-up of the myelin into spheres and the removal of the latter is certainly associated with the activity of macrophages, which appear in considerable number from the 7th day onwards (in the rabbit), apparently entering from the sheaths around the nerve (fig. 3). Spielmeyer (1929) considered that the myelin break-up at first took place within the Schwann cells which then transferred the break-down products to mesodermal elements. Others have considered that the Schwann cells themselves perform a phagocytic function, and their protoplasm may indeed occasionally contain degeneration products, but they certainly do not act as active phagocytes. The removal of the products of degeneration is effected by the macrophages, which become filled with debris,

forming characteristic large foam cells, whose nucleus and cytoplasm are hardly visible for the great number of granules which they contain. Often several macrophages lie close together forming the swellings ("fatty accumulations," "myelinspheres") which occur along the course of the old fibres. These masses decrease in size and number during subsequent weeks, being less prominent at 25 than 15 days. We find that in rabbit nerves examined 53 and 63 days after section macrophages are still numerous, and even at 79 and 85 days they are not uncommon. After 100 days, though still present, they are scarce. It is not certain how this removal takes place. In the intermediate stages (e.g., 34 days)



Fig. 3a

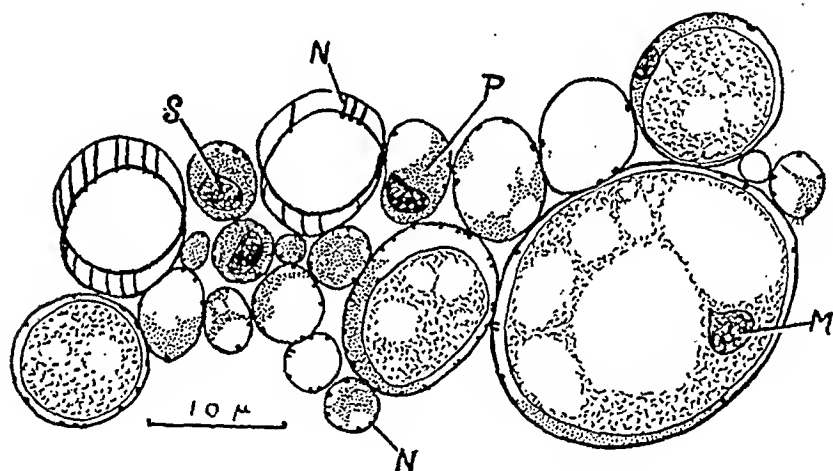


Fig. 3b

Fig. 3. *a*, longitudinal, and *b*, transverse sections of fibres in the peripheral stump 2 cm. distal to a suture made 25 days previously. Bodian's method. Macrophages have invaded the Schwann tubes, and the ends of some of the Schwann nuclei are pressed against them. Many fine nerve fibres run along the inner wall of each tube. *M*, macrophage nucleus; *N*, nerve fibre; *S*, Schwann nucleus; *P*, protoplasm of Schwann cell.

smaller macrophages, not crammed with fatty remains, may be very numerous. Perhaps these later invasions serve to remove the foam cells, which are so heavily laden that it is hard to suppose that they can recover.

Spielmeyer (1929) distinguishes by histological methods two stages in the break-up of the myelin, a Marchi stage from the 8th to about 21st day, and a Scharlach-red stage thereafter. During the first stage, which has its maximum about the 12th day after injury, the fats are imperfectly broken down and stain with osmium tetroxide or the Marchi stain, whereas in the 2nd stage they are simpler. All the times mentioned for the breaking up and removal of axons and myelin show considerable variation. Myelin is said to be removed more slowly

from large nerves than small and especially quickly in the neighbourhood of a large vessel (Spielmeyer). In cold blooded animals the break-up may be very much delayed, taking as much as 130 days in winter frogs (Möntekeberg and Bethe 1899).

Proliferation of the Schwann cells. The nuclei of the cells of Schwann divide mitotically, mainly between the 4th and 9th days after section (Cajal, 1928). We have seen mitoses in the rabbit as late as the 15th day. No counts have been made of the increase in number, but it certainly results in a multiplication by several times. It is not known whether the stimulus for division is a substance produced by the break-down of the axon or myelin. If such stimulating substances exist they cannot diffuse far, for when the tibial division of the sciatic of the rabbit is severed we have seen no multiplication of the cells of the peroneal, which lies close by. The fact that the multiplication occurs along the whole length of the peripheral stump is a remarkable instance of the stimulation of cell activity at a great distance from a lesion. However the effect does not extend across the neuromyal junction, since the nuclei of the end plate and of the muscle fibre multiply little if at all (see Tower, 1939).

The changes in the protoplasm of Schwann's cells have been described many times (v. Bünzner, 1891; Howell and Huber, 1892; Ranson, 1912; Spielmeyer, 1917, 1929; Boeke, 1916, 1935; Cajal, 1928; Nageotte, 1932) but there are many very important points about it which are still strangely doubtful. Most workers have supposed that no division of the cytoplasm follows mitosis, so that "a continuous protoplasmic cord is formed" (Cajal, 1928, p. 83). This cord at first occupies the spaces between the "accumulations of fatty droplets" which we have seen to consist of macrophages, but as the latter disappear the cord comes to form "almost the totality of the observable fibres" (*ibid.*, p. 85). Cajal supposes that this cord "although solid in appearance, is really a potential sheath whose central cavity has disappeared" (p. 86). In fact he believes that the protoplasm of the Schwann cells, which in the adult fibre exists as a sheet all over the myelin, becomes more voluminous when the fibre disappears from within it, and remains therefore as a thick-walled tube, with at least a virtual lumen. Further the cells of neighbouring segments become continuous, and thus make a strand, down the centre of which new fibres can grow. Other workers such as Boeke (1935) have agreed that the strand is continuous and syncytial, but have supposed it to be solid and to fill the entire tube. Boeke believes that the new fibres actually grow within its cytoplasm.

Examination of these bands after various types of fixation and staining has convinced us that it is incorrect to interpret them either as thick-walled tubes or, in early stages, as solid continuous masses of protoplasm. All have agreed that within the bands some longitudinal striation is visible, but careful study shows that this striation is not merely a fibrosity within a continuous mass but that the original tube is filled with a number of more or less separate, elongated cells. Bodian's (1936) stain can be made to colour these "Schwann fibres" almost specifically, especially in their later stages and after formol fixation (Holmes and Young, 1926).

The error of previous accounts lies in over emphasis of the syncytial nature of the bands. What really happens is that following the mitotic divisions each Schwann nucleus gathers a certain amount of protoplasm and becomes converted into a very elongated, fibrous cell. Each of these cells has a collection of protoplasm around the nucleus, and long tapering processes. In some cases these cells are in syncytial continuity with each other at the ends, but often it can be seen most clearly that the end of one fibre overlaps that of its neighbour. Similarly they may sometimes be continuous laterally, but very clear cracks can often be seen between them. Sometimes a fibre of one cell can be seen running past the nucleus of another. The exact details of the interrelations of such delicate protoplasmic structures are not at all easy to make out, since they are very liable to distortion by fixation. The important point is that they are fibrous entities, at least partly independent of each other, and not constituting a simple solid multinucleate cord of cytoplasm. Moreover they are able to move up and down within the tubes. This is shown by the fact that there are nearly always several Schwann nuclei collected at either end of one of the "accumulations of fatty droplets" (fig 3a). Cajal himself noticed that sometimes "these nuclei have level or slightly convex ends, which face the fatty droplets" (1928, p. 86). Such flattened nuclei always face the macrophage; while others, at the sides, give the appearance of pushing their way round the obstruction. During the early stages the nuclei are therefore more numerous at either end of these masses of macrophages than they are in neighbouring stretches. After about 40 days this irregular distribution becomes less marked, most of the nuclei having presumably moved to positions alongside the now somewhat shrunken masses. But some of the obstructions remain for 100 days or more as little clear spaces in the tubes, and these masses always have one nucleus pressed against each end.

Since we know that Schwann cells migrate, both in development (Harrison) and during regeneration in the tadpole's tail (Speidel) it is entirely reasonable to suppose that they also do so in the peripheral stumps during regeneration in Mammals. Recognition of this fact, however, implies some modification of current ideas of the "syncytium" of Schwann as a solid mass of protoplasm. Certainly there often are masses of Schwann protoplasm containing more than one nucleus, but the important point is to recognise that the cells are none the less fibrous entities, providing surfaces available for application to the new growing axons. In some cases indeed the ends of the Schwann fibres come to very definite terminations as clubs or balls, the result of frustrated migratory movements, causing the protoplasm to become piled up behind a mass of macrophages. The fact that these clubs persist until late stages perhaps implies that the protoplasm later becomes less fluid and its movements less active. Abercrombie and Johnson (1942) have recently found that the amount of migration from an explanted piece of nerve rises to a maximum at about 20 days after transection of a nerve, and thereafter falls, so that very few cells wander out from stumps which have remained uninnervated for a year.

Part of the confusion over the nature of the "degenerated" Schwann cells arises from failure to discriminate between the substance of the cell of Schwann,

the membrane of that name and the endoneurial sheath. The two last maintain approximately their original diameter during the early stages after interruption, being dilated with the products of axon and myelin degeneration. After fixation with Flemming's fluid it can be seen that these remains, and the macrophages, completely fill the cross section of the tubes, though with poor fixation the centre of the tube often appears empty. The Schwann protoplasm only gradually comes to fill the tubes, doing so at first between the masses of macrophages, and then in their places, the outer sheaths shrinking meanwhile. Probably the Schwann membrane (neurilemma) remains as a separate entity during degeneration, but after the disappearance of the nodes it is difficult to distinguish it with certainty from the endoneurium.

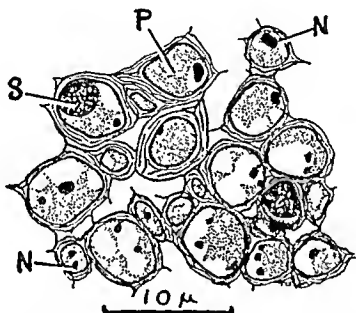


Fig. 4. Fibres from transverse section taken 2 cm. distal to a suture made 25 days before into a stump which had been left uninervated for 514 days. Bodian's method. The Schwann tubes are all much narrowed and filled with Schwann protoplasm (somewhat distorted by fixation). The nerve fibres are fewer and larger than those found after immediate suture (fig. 3b). Lettering as in figure 3.

After about 50 days, when many of the macrophages have gone, an isolated peripheral trunk is therefore made up of a number of "Schwann tubes," each of perhaps half the diameter of the original fibre, and filled with the elongated Schwann cells already described. In the smaller tubes there will usually be a single row of Schwann cells, so that a cross section after fixation with a good cytoplasmic fixative such as Flemming's fluid shows the tube to be filled with cytoplasm (figs. 4 and 5). In the larger tubes there may be several Schwann cells side by side, and divisions between them can be seen in cross-section. The naming of these structures is not difficult if we limit the term Schwann cell, Schwann band or band of Büngner to the actual protoplasm of the Schwann cell, and call the whole structure a Schwann tube. In the past the euphony of v. Büngner's name has led to its use indiscriminately for the whole tube and for the protoplasmic portion, so that it was not clear what was meant by the phrase "within the bands of v. Büngner."

The final state of a "sterile" or uninnervated peripheral stump is that of a thickened endoneurium, making tubes almost wholly filled by the protoplasm of the Schwann cells. These are so elongated that their nuclei often assume an S shape after fixation and when their fibrous protoplasm is stained they are exceedingly difficult to distinguish from nerve fibres. Any histologist seeing them might well be forgiven for supposing that autogenous regeneration had occurred in the stump. It is not known for how long they remain in this state; we have seen them very well developed 18 months after division of a nerve in the rabbit. It seems likely that, by a strange paradox, Cajal himself was deceived into the belief that some of these Schwann cells were axons. In some of his experiments he took special precautions to keep new axons out of the peripheral stump, but none the less believed that all of the stumps which he saw had become re-innervated. But his figures show that some, at least, of the "intertubal nerve sprouts" which led him to suppose that there had been an invasion of new axons, were in fact Schwann cells (e.g. his fig. 26 A). In his anxiety to deny the metamorphosis of Schwann cells into axons he failed to understand that they do change into fibres which very much resemble axons. But of course he was quite correct in supposing that the two are distinct. On the other hand he does suggest, very cursorily, the formation of separate Schwann cells, saying that "they break up their syncytial continuity, they narrow down and they flatten in order to apply themselves intimately to the connective membrane" (p. 91). He recognised that when seeing a space in this way at the centre of the tubes he might be dealing with an artefact. In fact in fully degenerated nerves every tube is filled with protoplasm, and it is into such filled up tubes that new fibres must penetrate; in earlier stages much larger spaces are available for them, at least in the larger tubes.

Re-innervation of the peripheral stump. We have seen that the outgrowing axons are led into the peripheral stump along the Schwann bands which have grown out across the scar. There is still no complete agreement as to how they grow down the peripheral stump itself. It is generally supposed that a fine bulb is present at the growing tip, but such bulbs are not often seen in preparations of regions of the peripheral stump which are in process of re-innervation. When they do appear they often seem to be the result rather of obstruction than of forward growth. The terminal portion of the axon is very fine, and probably normally there is only a very slight dilatation at its tip.

It is implied by some workers that the tips grow down within the protoplasm of the Schwann cells (Bielschowsky, 1935; Boeke, 1935, etc.; Nageotte, 1932, and perhaps Ranson, 1912). Others, such as Williams (1930), believe that the new axons grow at the edge of the sheaths (Schwann tubes) "rather than occupying the middle of the path." The confusion over this issue has arisen partly through failure to analyse properly the prior question as to the nature of the tubes, or to recognise that they are not in the same state throughout the whole process of degeneration of a peripheral stump.

Transverse sections of nerves sutured immediately after severance, and then fixed 15 or 25 days later show that while the tubes are still patent new axons

certainly grow along their inner walls and not down the centre of the tube (fig. 3b). During the re-innervation of the upper part of a peripheral stump after such a primary suture the Schwann bands are not yet fully formed, and the ingrowing fibres do not necessarily follow the Schwann protoplasm. Often 25 or more fine fibres can be seen running along the inner wall of a single large tube. On the other hand when new fibres grow into a stump which has been degenerated for a longer time, or into a smaller tube in a younger stump, they must come at once into more intimate contact with the Schwann protoplasm (fig. 4). Bocke and Nageotte give figures showing axons actually within the cytoplasm, but this does not of course prove that the tips actually bored their way down the tube in this way. Indeed it is difficult to imagine that growth could take place

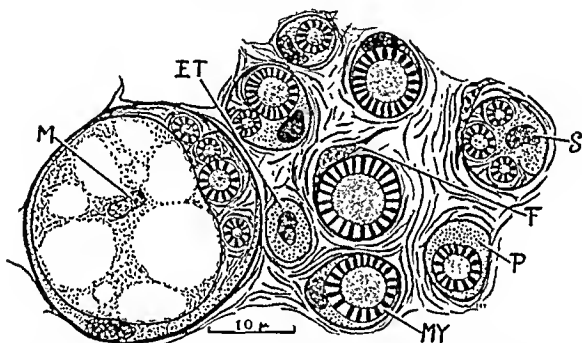


Fig. 5. *T.S.*, peripheral stump of rabbit's nerve severed 150 days previously. The stumps were left unsutured but a union was made by outgrowth; medullation has therefore proceeded rather less far than if the nerve had been sutured. Most of the Schwann tubes contain one or more fibres which are surrounded by Schwann protoplasm, *P*, and have myelin, *MY*. One tube, *ET*, remains uninnervated. Fibres are medullating even in the tube which still contains a macrophage, *M*. *S.*, Schwann nucleus; *F*, fibroblast. Zenker, Mallory.

within the protoplasm, which must be rather viscous. Possibly the axon secretes an enzyme which dissolves the Schwann protoplasm. But it is not difficult to find evidence that the newest part of the axon lies *on*, rather than *within*, the Schwann cell and that the protoplasm of the latter only later wraps around the fibre. For in many cases it can be seen that, as when axons are crossing the scar, the nerve fibre runs alongside the Schwann protoplasm, and, since this condition can be seen in many cases, there seems no reason to suppose that it is not the original condition of all the fibres. It must also be remembered that in some tubes there are several Schwann cells lying side by side, with interstices between them. The growing tip may creep down in such spaces and it may be that some of the appearances seen by Bocke and Nageotte were in fact not axons

within the protoplasm of a single Schwann cell but between that of closely apposed cells.

This Schwann protoplasm, which stains quite well when the cells are in the form of concentrated fibrous strands, ceases to stain soon after the tube in which it lies has been re-innervated, presumably because it spreads out as a thin sheet over the new fibre; for in later stages the nerve fibres are certainly surrounded by protoplasm (fig. 5).

After severance and immediate suture, then, many new sprouts invade each of the larger tubes. At later stages, however, thicker and much less numerous fibres are seen, only one or few of these occupying each tube (fig. 6). There can be no doubt that many of the fine fibres which are formed at first, later degenerate, and it would be of great interest to know what factors determine which axons remain and medullate. Possibly it is the activity of the Schwann cells, selecting one, or few, of the many fibres for re-medullation. Davenport, Chor

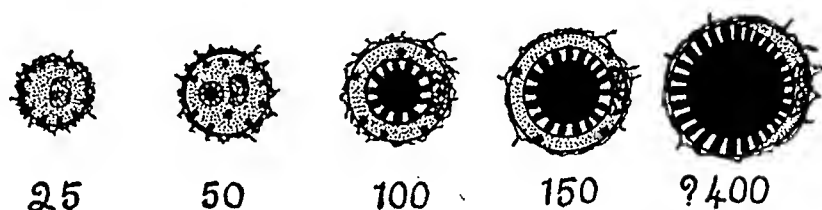


Fig. 6. Diagrams to show the progress of regeneration within a Schwann tube, with approximate dates of the various stages, as they would occur just distal to a good suture in the rabbit. At 25 days there are many new fibres at the edge of the tube. At 50 days one or two of these have enlarged, and are surrounded by Schwann protoplasm. At 100 days one fibre occupies the centre of the tube, others still remaining at the periphery. The times of final disappearance of excess fibres and attainment of normal diameter are uncertain. The Schwann protoplasm seldom appears as clearly as shown at 100 and 150 days since the axon and myelin, as they expand, soon almost fill the tube.

and Dolkart (1937) have shown that 6 months after suture of the sciatic nerve of the dog there are fewer fibres in the peripheral stump than in a normal nerve. But Dogliotti (1933) and others have found that when a small nerve is sutured into a large one, there is an excess of fibres in the peripheral stump.

In cross sections of the peripheral stump of rabbits' nerves sutured 100 days previously we have seen one or few large fibres in each tube, these being already quite heavily medullated. Around them, however, there still remain several small fibres, at the edge of the tube. Though the total number of fibres seems somewhat less than at say 25 days it is clear that the reduction of the excess is not a rapid process. However these peripheral fibres had disappeared from stumps examined one year after suture. It is possible that the fibres which obtain preference are those which increase in diameter more rapidly. This in turn may depend on the number of successful branches put out from the central stump. If many branches from any one central fibre gain entrance to the peripheral stump it is unlikely that they could all rapidly increase in diameter.

Therefore they do not medullate, and the functional result is not impaired by the connection of one fibre with various end organs. It is, of course, well known that more than one fibre may persist in the peripheral stump connected with a single central fibre (see Howe, Tower and Duel, 1937), but the mechanism suggested above may minimise the number of such connections.

When a secondary suture is made, the peripheral stump having been allowed to degenerate until the tubes are filled with Schwann protoplasm, there seems then to be less opportunity for new fibres to penetrate into the peripheral stump than when open tubes are present (fig. 4). Therefore there are fewer fibres in stumps after secondary than after primary suture at least during the early stages of re-innervation. (See p. 364.)

Rate of regeneration of nerve. On account of the emphasis which has been given to the process of penetration of new axons into a denervated stump it has been usual to consider that the "rate of nervous regeneration" is synonymous with the rate of advance of the tip of the axon. It is important, however, to remember the postulate laid down in the first sentence of this review, namely, that "regeneration" of a nerve implies its recovery to a state in which it is able to conduct impulses which shall be effective for function. The state whose rate of advance down the nerve is of the most interest is therefore not that of the presence of the axon tips, but of a sufficient number of fibres in a condition which we may call that of *functional completion*. Functionally completed fibres are those that have undergone a process of maturation, including increase of diameter and medullation, so that they are able to carry effective impulses. There is no presumption a priori that this state of functional completion advances at the same rate as the axon tip, it might advance slower or, after starting later, faster. Evidently it is necessary to investigate separately first the rate of progress of the tips and then of the advance of functional completion. Some methods of measuring "the rate of regeneration" will measure the rate of the tips alone; for instance, histological examination of the point reached by the foremost fibres. Other methods, which depend on recording the return of function after lesions at various levels give an estimate of the advance of functional completion. It was indeed comparison of estimates of the rate of regeneration made by various methods which led to a realisation that different methods do not all measure the rate of advance of the same entity (Gutmann, Guttmann, Medawar and Young, 1942).

Rate of advance of the axon tips. The rate of growth of the tip of the axon varies very much with the conditions (see Williams, 1930). In the scar between stumps Cajal estimated the rate as 0.25 mm/day. But the fibres spin down the peripheral stumps very much faster than this. By direct histological observation Cajal estimated their rate in cats, dogs and rabbits to be 3-4 mm/day, and we have confirmed this in rabbits by a combination of histological and experimental methods. In our experiments the nerve was tested by pinching under light anaesthesia. If the pinches are made first distally and then progressively up the nerve, the animal gives reflex responses when the innervated region is reached. This is found to give a sensitive test of the point reached by the tips of the fastest-growing

fibres, and subsequent examination with Bodian's stain shows that only very few, and very fine, fibres need be present to give the reflex.

By making such examinations on different rabbits at various times after suture it is possible to plot the advance of excitability down the nerve; and the regression coefficient of distance reached on number of days gives an estimate of the rate of growth. We found that after severance and primary suture, whether by stitches or by the plasma method, fibres grow at 3.45 ± 0.16 mm/day in the rabbit. There is a latent period of 7.3 days before any fibres appear in the peripheral stump, and this figure also lies within the limits estimated by Cajal and others using histological methods.

In cases in which the nerve was not divided but the axons were interrupted by thorough crushing with fine forceps, the rate of growth was found to be higher, namely, 4.36 ± 0.24 mm/day. This type of injury interrupts all the axons, which undergo Wallerian degeneration, but the connective tissues maintain continuity, and provide optimal conditions for outgrowth. The latent period is shorter, 5.2 days, than after suture, which is not surprising, but it is interesting to find that the axons actually advance faster along the peripheral stump than they do after suture.

Further experiments have established that in the rabbit there is no great difference between the rate of advance of the axon tips in the different divisions of the sciatic nerve. Nor is there any difference in the rate after lesions made high up in the thigh or below the knee. However in the rabbit the differences in distance from the cord involved in such experiments cannot be made great, and it is possible that in man this problem of the rate of outgrowth at different levels is more complex. Several reasons might produce an apparently slower growth at the periphery. 1, the greater distance from the cell body; 2, the growth rate may decline as the length of regenerated nerve becomes greater, this decline being due either *a*, to a falling off with time of the power of outgrowth from the cell (the *vis a tergo* of Held), or *b*, to an increasing resistance of the tissues of the peripheral stump, since in the more distal parts of the nerve the Schwann protoplasm will have more completely filled the tubes (p. 345).

In our experiments we have not seen any evidence that the rate of outgrowth of the axon tips declines with the distance from the lesion, but we have followed them only for about 10 cm. There is no reliable evidence that the rate of growth of fibres, or indeed their medullation, can be influenced by external treatments, though various claims have been made. Deineka (1909) claimed that the early stages of regeneration were much more rapid when the external temperature was raised.

On account of the complications introduced by the use of functional recovery as a criterion of regeneration (see below) it is not possible to say whether there are differences in the rate of growth of different types of fibre. Evidence from studies of regeneration in the sympathetic system indicates that outgrowth of pre- and post-ganglionic fibres takes place at rates not greatly different from those of spinal nerve fibres (Lawrentjew, 1925; Machida, 1929; De Castro, 1930; Tower and Richter, 1931). De Castro believes that the postganglionics grow more slowly than the preganglionics, but none of the evidence is decisive.

Maturation of the new nerve trunks. The very thin nerve fibres which are first laid down are certainly able to carry impulses, but the conduction rate is very low. Before a nerve can carry volleys at sufficient speed and synchronous enough to produce, for instance, motor function, the new fibres must thicken and become medullated. Very little is known of the process of thickening, whether it is dependent on the Schwann cells in the peripheral stump, or what determines the final diameter reached. Presumably there is some correlation with the nature of the nerve cell body, and especially its size. The simplest hypothesis is that the process of growth of the axon is essentially a flowing out from the intact stump and that the larger the parent axon the greater the flow, so that new fibres come to have sizes similar to the old. But during regeneration the parent axon has divided into very many fine branches, and it has long been known that at least two new axons attached to a single fibre may persist for long periods after suture (Oshorne and Kilvington, 1909; Howe, Tower and Duel, 1937) see however p. 345.

The increase in diameter of the fibres certainly advances progressively down the nerve. Within the first 10 mm. distal to a good suture point the largest fibres reach a diameter of half that of the largest fibres in the central stump by the 25th day. In a rabbit examined on the 82nd day after suture the axons in this upper region reached a diameter equal to that of the central fibres, 6μ in these preparations. At a point 140 mm. distant from the suture however the largest fibre seen was only 1.5μ . Yet the fibres must have reached this more peripheral point on the 48th day (see p. 345). That is to say, they are much smaller than the fibres in the first 10 mm. were on the 25th day, although they have been laid down for 10 days longer than the latter.

The rate of increase of diameter may not therefore be the same at all points along a peripheral stump. This would perhaps be expected if it depends on a flowing out from the central stump.

The process of medullation is also progressive along the nerve. Speidel (1932) has shown that during regeneration in the tail of the tadpole medullation advances distally by the migration of Schwann cells from unmyelinated to myelin-emergent fibres. It is not certain however that the process in the main nerve trunks of mammals is similar to that which he observed in the newly formed nervules which develop in the tadpoles. In particular it seems likely that in mammals the new fibres are in contact with Schwann cells from their earliest stages, though as we have seen the cells may also migrate along the fibres.

Clark and Clark (1938) observed the process of medullation of fibres growing in chambers in the ear of the rabbit, and saw several segments medullating at once. The general downward advance of medullation has been noticed by several workers (e.g., Howell and Huber, 1892; Lewis and Kirk, 1916). Sanders and Young (1942) have found clear evidence of a well defined front of medullation.

There is scattered evidence about the time at which medullation begins. Speidel (1932, p. 311) reports that in some cases "axon extension and myelin unit addition often proceed together, *pari passu*", but on other fibres medullation appeared only later and advanced with a rush. Lewis and Kirk (1916) found that medullation only begins when "the regenerated axis cylinder has reached the age

of about 5 or 6 weeks''. This is certainly an overestimate. In the rabbit 15 days after a suture we have observed that there are a very few finely medullated fibres in the scar region. Fifteen days after a nerve had been crushed medullation was found in the first 10 mm. below the point of injury, but not at more peripheral levels, although by this time the axon tips had reached to a distance of 42mm, having presumably first entered the distal stump on the 5th day (see p. 345). At this level, therefore, the very beginnings of medullation appear about 8 days after the fibres have been laid down (see Sasybin, 1930; Hentowa, 1933); there is no certainty that this interval will be the same in more peripheral regions, especially since we have seen reason to suspect that the increase in axon diameter becomes slower towards the periphery. Davenport et al. (1939) produce some evidence that there is no long delay in medullation of fibres towards the periphery. We have found that 100 days after suture in the thigh of the rabbit medullation is much less complete in the posterior tibial nerve than close to the lesion. It seems possible therefore that there is progressively slower medullation in the more distal regions.

The first sheaths to be laid down are very thin. Hentowa (1933) has described the way in which they thicken and become provided with nodes and incisures. It is not known whether node lengths similar to those of the original fibre are re-constituted, nor whether the great number of Schwann nuclei formed early in regeneration later becomes reduced until there is only one nucleus in each internode. We do not know what controls the final thickness reached by the myelin, presumably it depends on the axon diameter (see Duncan, 1934; Schmitt and Bear, 1939). The whole process of maturation is not completed for a considerable time, and the later stages have never been studied in detail. It can be shown that the power to produce medullated fibres is dependent on some property of the central rather than the peripheral stump. We (Simpson and Young, unpublished) have removed the splanchnic ganglion and sutured the central end of one of the spinal nerves (D 13) into the postganglionic trunk of the anterior mesenteric nerves. Normally these contain only very few medullated fibres, but 100 days after the operation numerous medullated fibres were present. However, they were much smaller than they would be after a corresponding suture in a spinal nerve. The nature of the peripheral stump therefore has some influence on the final diameter which is reached.

The converse experiment shows that this influence is not decisive. The central ends of anterior mesenteric nerves, still attached to the ganglion, were sutured into the peripheral stump of D 13; 100 days later stimulation of the nerve produced contraction of its muscles, but sections showed that the fibres were all very fine and unmedullated. Clearly therefore there is some property of sympathetic neurons which makes them unable to produce large fibres such as form on the cut end of some somatic fibres. The difference cannot be simply a matter of size, perhaps it is related to the number and length of dendrites (which are large in sympathetic cells) or to some other factor.

Very little is known about the time at which nerve fibres recover their original diameter, or the extent to which the original pattern of fibre sizes is reproduced.

Grecnman (1913) found that 100 days after crushing the peroneal nerve of the rat the largest fibres in the distal stump were smaller than those of the control side. Weiss (1937) has shown that in Amphibia the new fibres assume a variety of diameters, but not whether these are distributed as in a normal nerve.

Gutmann and Sanders (unpublished) have measured the diameters of medullated fibres found in the peripheral stump at times up to one year after simple suture of the peroneal nerve of the rabbit. In a normal peroneal, at the level considered, the diameter of the largest fibre is 19.9μ and in the regenerating nerves a very few fibres of this size were found at 200 days, rather more at one year. But the histogram of figure 7 shows that the majority of the fibres had not yet recovered to the normal size. Still more important is the fact that there is no trace of the separate group of large sized fibres, presumably the α fibres, which can always be detected in counts made on normal nerves. At 200 days the muscles innervated by the nerve had been active to some extent for about 140 days, though, as explained on p. 367 the functions were still very far from perfect. The sensory recovery, starting much later, was even less complete (p. 368). It cannot yet be decided whether these imperfections of function are a result of the incompleteness of the restitution of the fibre-size pattern of the nerve, or whether other features, such as inadequacy of number of fibres, are concerned. Study of even longer periods of recovery is now necessary. It is clear that the early period of regeneration, which has been so much studied, includes only a part of the process, and that we do not even know whether it is ever completed. There is, however now some evidence on the problem if we may conclude that the increase of diameter of the regenerating fibre depends on outflow from the central stump, but that the flow is impeded when outgrowths from large fibres enter small peripheral tubes. After suture of a mixed nerve, therefore, many of the outgrowing fibres will be in the same position as those of the spinal nerve entering the tiny tubes of a postganglionic trunk in the above experiment, and they will be able to increase in diameter at best only very slowly. Correspondingly functional recovery is seldom (? never) perfect after suture, though it may be nearly so after a nerve has been simply crushed, so that each fibre can return into its original tube.

In fact each Schwann tube retains after degeneration the character of the original fibre which is perhaps most important for functioning, namely, its diameter, relative to other tubes. Each tube can therefore only regenerate fully if it is innervated by a fibre like the original one. We may suspect that extension of the observations of Gutmann and Sanders will show that the fibre size distribution of a nerve is never recovered after suture, and the more delicate functions must remain correspondingly imperfect.

Rate of advance of functional completion. If a certain degree of maturation of the new nerve fibres is necessary before functioning can take place interesting factors are introduced to the problem of the rate of advance of regeneration down the peripheral stump. Recovery of, say, the sensation of touch, or the reflex movement of a muscle, will then depend not on the presence of unmyelinated axon tips but of fibres matured to whatever level may be necessary. It therefore follows that the 'rate of regeneration' which is significant for studies of recovery

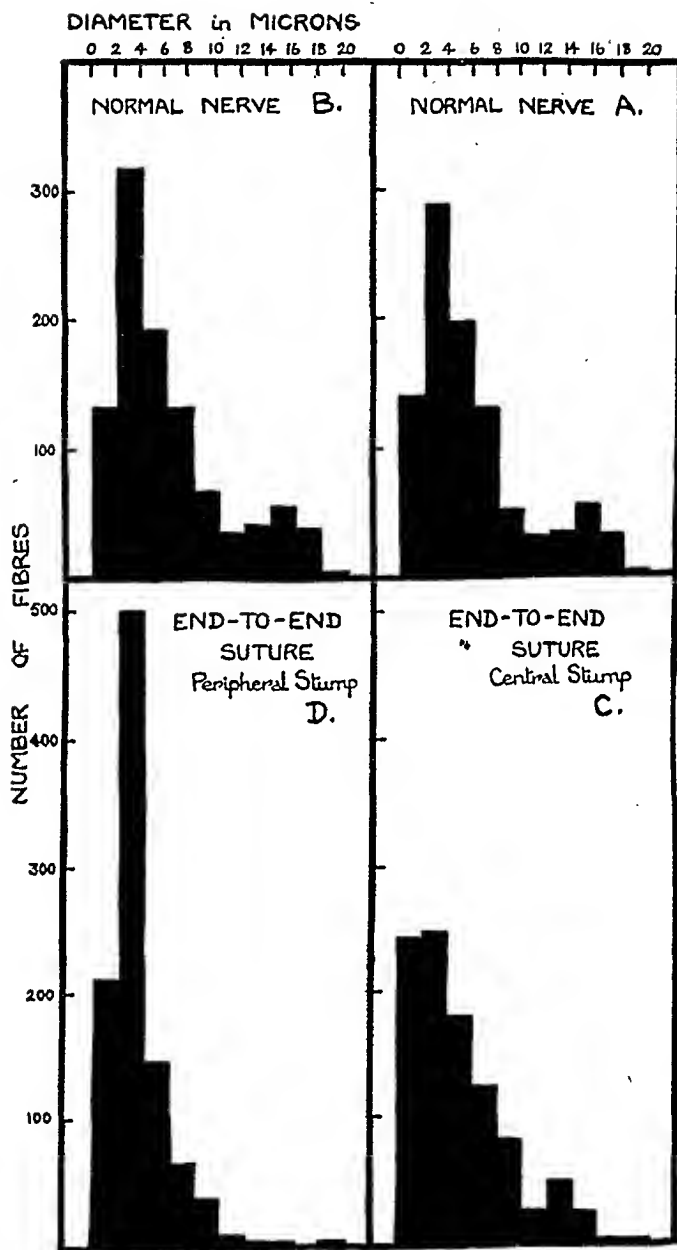


Fig. 7. Histograms showing the numbers of medullated fibres of various sizes in the peroneal nerve of the rabbit expressed as per thousand of those counted. A and B, normal nerve at levels corresponding to C, 2 cm. above and D, 2 cm. below a suture made 200 days previously. (Figure kindly supplied by Mr. F. K. Sanders.)

will not be that of the tips, but of the functionally completed fibres. We have seen that the tips advance at 3.5 mm/day after suture, 4.4 mm/day after crushing, and that medullation only begins some time after the tips of the fibres have been

laid down. Presumably there is a still further interval until the fibres have thickened to that degree which is necessary for functioning, that is to say, to a state of functional completion. Once this state has been reached function will become possible in the region immediately adjacent to the suture. But what will be the subsequent rate of advance of this state or level of maturation? Will it be the same as that of the axon tips, or faster, or slower?

Measurement of the rate of advance of the region of the nerve which has regenerated to this level of functional completion can be made by comparing the times necessary for recovery after lesions at various levels. We have made this estimation in the rabbit both for sensory and motor recovery (Gutmann et al., 1942). The motor function studied was the spreading of the toes of the rabbit, produced by the small peroneal muscles, innervated by the peroneal nerve. This nerve was therefore interrupted at various levels, and the times of recovery noted. Assuming that functional completion advances down the nerve at a constant rate the regression coefficient of distance from lesion to muscle on time taken for recovery then gives the rate of advance of the regenerated region. The results obtained were 3.1 mm/day after crushing the nerve, and 2.6 mm/day after severance and suture, with latent periods before the advance begins of 21 and 37 days. This means that after suture the nerve in the uppermost portion of the distal stump reaches a state in which it can function after 37 days, and this condition then advances down the nerve at 2.6 mm/day.

Similar figures were obtained by observing the time necessary for sensory recovery to reach a given degree at a determined point after lesions at various distances. The criterion used was the complete return of sensitivity to pin prick over the whole surface of the part of the foot rendered insensitive by interruption of the peroneal nerve. The rate of advance of functional completion given by this method was 2.5 mm/day after crushing, with a latent period of 19 days. After suture this method gave a rate of 2.4 mm/day. However the variability between the cases was very large.

In spite of considerable individual variations, these quite independent estimates agree well, and seem to show that the processes of increase in diameter and medullation, which complete the regeneration of the nerve, advance down it more slowly than do the axon tips. Before accepting this conclusion we must consider some possible complications. First, the experiments in which the nerve was pinched were shown by histological examination to give an estimate of the distance to which a few of the fastest-growing fibres had reached. But sensory and motor function will only appear when numerous fibres have recovered, so that the above estimates are of the rate of advance of the functional completion, not in the fibres which recover fastest but in those of, perhaps, average velocity.

Secondly, the apparently slower rate of advance of functional completion might be due to the fact that there is a delay in the end-organ before function returns, and that this delay is greater after more distant lesions, since they impose longer periods of atrophy. To control this possibility the injury close to the muscle was made twice, in such a way as to ensure that the total period of denervation

was the same after the high and low lesions. The rate of advance of functional completion calculated from these experiments was 3.5 mm/day, hardly higher than that given above for crushed nerves.

We may conclude that this factor is not important, but there is a third possibility, namely, that the degree of functional completion necessary may be greater the further the lesion is from the end-organ. Over a short stretch of nerve relatively incomplete fibres may conduct impulses sufficiently synchronous to produce, say, visible contractions, but longer stretches may require larger fibres and more myelin. If this factor is important not only are the above estimates of the rates vitiated, but also we have the awkward situation that there is no definite "rate of advance of regeneration," since the degree of recovery of the nerve which constitutes regeneration will vary with the distance from the lesion to the end-organ.

It seems probable that none of these three factors is very important in the experiments in the rabbit, where the distance from lesion to end-organ is not great, and our conclusion that functional completion advances more slowly than the axon tips is probably sound. In Man, where distances are greater, such factors may be very important. Further, the degree of maturation which is necessary for functioning may differ with the complexity of the function concerned. Facilitation, whether central or neuromuscular, requires that impulses arrive at a certain frequency in each fibre. Delicate movements or discrimination depend on appropriately timed volleys. This may be part of the reason for the order in which functions return in the skin of Man, namely, approximately, pain, touch, cold, warmth. Fine touch recovers very late and often imperfectly. It is even possible that the unpleasant quality of the pain felt during recovery is due to the slow conduction of the impulses and their scattered arrival in the centres.

Finally it must be remembered that we have assumed throughout these calculations that the rate of advance of functional completion is constant along the whole peripheral stump. This may be so for the short distances used in the above experiments, but for reasons explained on pp. 347 it may be that the increase in diameter, and hence of medullation, occurs more slowly at great distances from the lesions. This factor would tend to make the rate of regeneration in large animals such as man appear to be slower than in the rabbit.

All of these considerations show that the concept of rate of regeneration is very far from being as simple as it may at first sight appear. We must beware above all of assuming that there is any single "rate of regeneration." It is certainly not possible to calculate this rate in man simply by dividing the distance from the lesion by the time taken for recovery. It is this crude method which has produced the very low estimate (1 mm/day) which circulates so widely. But from careful observations, such as those of Trotter and Davies (1909) it is clear that in man the advance of functional regeneration may occur at rates as high as 2.4 mm/day. By careful selection of suitable cases, and by study of the exact times at which the functions of muscles return, it should be possible to obtain accurate estimates of the rate of advance of recovery of the nerves of Man to at which they can mediate the various functions. In some cases this

prove to be as high as 2-3 mm/day, though it may be much lower when the criterion used is full recovery of a delicate function.

Regeneration of the dorsal roots and spinal cord. Although functional restoration is rare after lesions of the dorsal roots or within the C.N.S. there is evidence to show that outgrowth of new fibres can take place in these situations. The later failure of these sprouts is presumably the result of the difficulty of finding suitable pathways, which results from the complexity and special histological composition of the centres. Paskind (1936) has confirmed the finding of early workers that fibres can grow out from the cut central end of a dorsal root. He cut the cervical and thoracic roots in the cat, apparently leaving the stumps unsutured. There was typical degeneration and cell proliferation within the stump remaining attached to the cord, but only as far as the pia glial membrane. The regenerating fibres, emerging from the stump attached to the ganglion, also reached only to this level and entered the cord only very rarely, usually alongside blood vessels. On the other hand other workers have found that at least some regenerating fibres can enter the cord, and the exact conditions under which they can do so still require to be investigated. Further work in which the severed ends of the roots were held as close together as possible, perhaps with the aid of plasma, should throw further light on the problem.

Within the central nervous system itself four possible types of regeneration may be considered.

a. Replacement of destroyed nerve cells.

b. Establishment by damaged neurons of substitute connections.

c. Re-medullation of tracts temporarily damaged.

d. Re-establishment of connections by regenerative outgrowth of axons.

a. It is almost certain that regeneration of this type cannot take place in vertebrates. There is a considerable literature on the problem of whether mitotic figures appear in the nuclei of neurons after punctures, resections or infections (see Rossi and Gastaldi, 1935, for summary). The general opinion is that although the nucleus of a neuron may attempt and occasionally even complete a division, yet no satisfactory cleavage of the whole cell ever takes place. On general biological grounds it is perhaps hardly to be expected that the intricate morphogenetic processes necessary to produce the finer details of the higher nervous centres could be reproduced in the adult.

b. This is a very doubtful sort of functional regeneration, typified by the reaction of cells in the cerebral or cerebellar cortex which, when their axon is cut off, enlarge one of their dendrites into a recurrent fibre leading to the more superficial cortical layers (see Bielschowsky, 1935). Cajal (1929) suggests that by this means it is ensured that the impulses of the cell are not wholly 'wasted', and it cannot be excluded that such new connections might have some importance in re-education. But it is impossible to say more until we have further information about the modes of action of the cells in the cortex under normal and abnormal conditions.

c. Re-medullation, though interesting and important for recovery from pathological conditions, falls outside our present scope.

d. The possibility of functional regeneration within the central nervous system

of mammals has now been definitely shown by the work of Sugar and Gerard (1940). It has long been known that in Teleostean fishes and Amphibia full functional regeneration can take place even after section of the adult spinal cord (see e.g. Lorente de N6, 1921; Tuge and Hanzawa, 1937), and that in Mammals it can occur if the cord is cut during intrauterine life (Gerard and Grinker, 1931).

Indeed there is much evidence that the cut central axons are capable of putting out regenerative processes such as those formed in peripheral nerves. Sugar and Gerard have now found 13 cases in which this power has produced functional regeneration in adult rats. They severed the thoracic cord, taking precautions to avoid damage to the blood supply of the isolated section. With careful bladder treatment they obtained good survival, and functional regeneration was shown, during the second month, by voluntary climbing, placing and seeking movements of the hind limbs. "At sacrifice, stimulation of the brain stem produced hind leg movements". Fibres were shown histologically to have grown across the scar, helped in some cases by pieces of nerve and muscle which had been planted there.

Therefore the rarity of re-establishment of function in the C.N.S. must be due to special factors. Either 1, to the scar tissue not providing a suitable medium for growth, or 2, to the degenerating fibres not offering suitable pathways, perhaps because of the absence of Schwann cells³ or 3, to the great difficulty of establishing, in so complex a system, any appropriate connections by random regenerative outgrowth.

The alleged unsuitability of the scar tissue can hardly be a complete barrier to regeneration. Fibres, apparently freshly growing, were seen by Rossi in the scar 250 days after cord section in the cat. Spatz (1930) and others have given careful consideration to the peculiarities of the glial degeneration, as a possible factor preventing satisfactory regeneration. This whole aspect of the problem is a very promising one and would be worth further investigation. It is not clear whether the main difficulty experienced by the fibres is in penetrating from the scar to the distal stump or in traversing the latter. This is a point which it would be worth while trying to clear up. It is possible that part of the difficulty is mechanical and that better results could be obtained by careful approximation of the stumps, or perhaps by further development of artificial bridges of muscle or nerve such as were used by Sugar and Gerard.

Re-innervation of end organs. After denervation the sensory and motor endings do not in general disappear, at least for the first few months. They may undergo various changes, but if re-innervation is moderately rapid the new fibres will find at least an outline of the old organs when they reach the periphery. It is probable that this maintenance of the specific end organs determines the course of recovery to a large extent. We have seen that there is no reason to suppose that the outgrowing nerve fibres are in any way sorted or shunted into appropriate paths; similarly there is no evidence of the attraction of any fibre back to its end-organ. As a result many of the connections formed must be unsuitable ones, and it is to compensate for this that excessive innervation occurs at all

³Sugar and Gerard found Schwann-like cells along some of the fibres in their scars.

stages of regeneration. Presumably many of the connections made at first are aberrant, such as sensory fibres in motor end-plates; for it has been shown by several workers, and recently most clearly by Weiss (1935), that sensory fibres can make connection with motor end-plates. Similarly from experiments such as that described on p. 348 it is known that postganglionic sympathetic nerve fibres can make connection with striped muscles. Weiss joined the central end of the 9th dorsal root of toads into muscles transplanted into the back, and hence fully denervated. After a while these muscles contracted when the nerve of this mononeuronal arc was stimulated electrically, but never on stimulation of the sense organs at its other end. Weiss interprets this failure in terms of his theory of resonance, though it cannot be excluded that his success with electrical and failure with sensory stimulation was connected with the number of fibres stimulated and the synchrony or frequency of their impulses. For our present purpose the significant point is that sensory fibres are able to form functional connections with end-plates. But it should be noted that the endings which Weiss found were "largely atypical." After introduction of a motor root into a muscle more effective functional connections were made.

It is not known what is the proportion of such atypical connections which are normally formed during regeneration. During the re-innervation of muscle we have observed that usually only one nerve fibre runs to each end-plate; occasionally two may do so. When a suture is made with a mixed nerve it would seem that a very considerable number of connections must be irregular and that these would monopolise many of the end-plates, unless they afterwards degenerate and are replaced by others. It may be that grossly atypical connections, as of sensory fibres with motor end-plates, are absorbed in this way, but there is certainly no efficient mechanism which ensures that lesser irregularities are corrected. Many cases are known in which motor nerve fibres come to make atypical connections, and continue to produce action of the muscles under absurd conditions (see Ford and Woodhall, 1938).

If there is a process of absorption of some of the fibres which make unsuitable endings it must be another instance of that strange "trophic" influence by which functioning fibres are maintained while non-functional ones are destroyed. This seems to our practical sense to be obvious and fit, but we must realise our ignorance of the machinery which produces this business efficiency of the body. It is not enough to say simply that the redundant nerve fibres suffer an atrophy "from disuse". The extent of the re-adjustments which may take place after disturbance of the peripheral nerves is greater than is often supposed. Greenman (1913) cut the peroneal nerve of rats and found a reduction of 16 per cent in the number of fibres in the peroneal nerve of the *opposite side* of the animal.

Though there is, therefore, no evidence of attraction of fibres back to appropriate endings there is, nonetheless, a definite influence of the collaterals on the ingrowing fibres, leading them to branch in ways which are appropriate to the formation of sensory and motor connections. Fibres which have grown without branching down the nerve trunks begin to divide when they reach their proper uses and end-organs. Although we have very little information as to the cause

of this branching there is no evidence to indicate that it is due to any influence other than the obstruction of forward growth, which produces branching at the point of entry to the peripheral stump (Cajal) or anywhere else that it occurs.

There is evidence, however, that new fibres entering muscle can act reciprocally with the latter to produce new motor end-plates, and similar phenomena are seen in the skin. Grigorieff and Lawrentjew (1930) have described the formation of structures like end-plates where nerve fibres meet myoblasts in cultures in vitro. Similarly new end organs may be produced where nerve fibres enter the skin. It would be impossible therefore, with our present information, to deny that there may be some special influences of the terminal regions on the outgrowing nerve fibres and vice versa. There are facts which seem to show that re-connection is not simply the outgrowth of new tips down the old pathways until they arrive at the existing end-organs. And yet a considerable part of the process consists of little more than this, at least for the period during which the old organs remain to a large extent intact after degeneration of their nerves.

Re-innervation of motor end-plates. Tello (1907) and Boeke (1916, 1917, summarised 1921 and 1935) have given beautiful accounts of the behaviour of end-plates after denervation, but there are still some points of great interest which are in doubt, largely for lack of proper quantitative treatment. After severance of the nerve to a muscle there are changes in the protoplasm of the end-plate, and after about six months it may completely disappear (Tower, 1939). There is still some uncertainty as to whether there is any multiplication of the end-plate nuclei. Apparently the central nuclei of the plate atrophy, whereas the peripheral ones may multiply. There are some reports of mitosis, but many reliable workers have failed to see it (see Boeke, 1935). Boeke believes that some nuclei may divide amitotically, and he says categorically that "man findet bei fortgeschrittener Degeneration immer mehr Kerne innerhalb der Sohlenplatte als im Anfang der Degeneration" (1935, p. 1013). However after long periods of denervation the nuclei of the end-plates can no longer be distinguished from the subsarcolemmal nuclei of the muscle fibres; in fact, the end-plate has completely disappeared (Tower, 1939).

When new fibres return to the muscle they branch out, both within the old end-plates, and also at other points among the muscle fibres. Boeke (1916, p. 67) is especially convinced of the formation of new end-plates in this way as collaterals. At first the new innervation is abnormal, and in particular it is excessive, and Boeke gives a long series of figures illustrating this abnormality and its gradual reduction. Already during the first $1\frac{1}{2}$ –3 months after operation there were found to be some apparently normal end-plates, but it was not until more than five months after suture that the majority approached the normal state.

It would be interesting to analyse this abnormality to discover to what extent it is correlated with the irregularities of function found during the early part of recovery. It is difficult, however, to assess the significance of the aberrations in shape of the fibres in the endings, since we understand so little about the meaning of the forms normally observed. Moreover at the time Boeke's study was made too little was known about the rate of regeneration of the nerve fibres, or of the

significant variables of muscle functioning, for detailed correlations to be made. There is still much to be done in this respect, but since the rates of regeneration are now known accurately in the rabbit it has been possible to make some observations of structure and function during the critical period after the first arrival of fibres at the muscle (Gutmann and Youag, unpublished).

The peroneal nerve was crushed in a series of rabbits 25 mm. from its point of entry into *m. peroneus secundus*, which produces abduction of the fourth toe (p. 367). After this injury, fibres begin to arrive back near the end-plates after about 13 days, allowing 5 days for the initial delay, 6 days for growth down the trunk at 4.4 mm/day and 2 days for growth within the muscle. Histological examination on the 14th day confirmed the presence of fibres in the intramuscular nerves, and their absence from the end-plates. Muscles taken on the 16th and 18th days also showed no fibres in the end-plates, so that there is evidently a short period of delay before the fibres succeed in penetrating into the plates. Immediately they do so, however, they become able to excite the muscle, for in another muscle, examined on 18th day, faradic stimulation of the nerve produced a contraction, and some of the end-plates contained fibres. The reflex contraction of the muscle, causing spreading of the toes when the animal is suddenly lowered, appeared first on 23rd day, and about this time the nerve fibres in the muscle begin to be thinly medullated.

The variables are so related that it is impossible to be certain what is the cause of the short delay between appearance of indirect excitability and reflex function, but there is no reason to suppose that it is not due mainly to the need for the addition of further innervated end-plates. Other factors operating may be maturation of the end-plates themselves, such that they are able to transmit with less delay, and improvement in the power of the nerve fibres to transmit impulses at appropriate frequencies.

The connections made with the end-plates on the 18th day were very simple. The fibres were still very thin, though usually somewhat thickened just before their entry to the plate, as if they had been obstructed. Within the plate very few and excessively fine branches had been formed. During the subsequent period much richer branching and thickening of the knobs occurs, so that by 27th day the innervation could already be called excessive. It would be very interesting to study the mechanical, pharmacological and electrical responses during this period of innervation, and especially the responses at the end-plate (see Eccles, Katz and Kuffler, 1941).

It is possible, then, that the additional branches and thickenings which are formed within the end-plates during the period after the first arrival of fibres are responsible for the increasing strength of movement which occurs (see p. 367). But it is just as likely that this improvement in function is due to the arrival of further fibres at end-plates, and to increasing medullation. In any case it is clear that under these conditions there is no delay longer than 10 days after arrival of fibres before functional connections are made. From this study of quick re-innervation in the rabbit it is not of course possible to speak definitely about the processes of recovery following sutures after longer periods of atrophy such as

occur in man, and which probably involve complete disappearance of the end-plates. There is no evidence available to tell us whether under such conditions a long delay occurs while the newly-arrived fibres are forming their end-plates.

Degeneration and re-innervation of muscle spindles. Tower (1932) has made a careful study of the degeneration of the spindles in the interosseus muscles of cats following section of the dorsal and ventral root innervation or both. After severance of the nerves in the limbs the changes in the muscle fibres in the spindle are similar to those in the extrafusal muscle fibres. There is first a process of atrophy and then, after about six months, degeneration begins to set in, so that after the denervation has been maintained for a year, only a few, highly modified spindles remain. The capsule becomes thickened and shrinks down, obliterating the periaxial space around the remains of the muscle fibres.

Following section of the ventral roots alone Tower found that there was full degeneration at the poles of the intrafusal fibres, but that the equatorial region, being still provided with its sensory fibres, maintained its structure, except for some changes in the contractile substance of the fibres. Conversely after dorsal root section the poles were unaffected, but in the equatorial region the regular arrangement of the nuclei was lost and typical cross striation appeared. These very important observations show most clearly how the presence of a nerve fibre is able to maintain the characteristic structure of the tissue it innervates.

Re-innervation of muscle spindles has been studied by Huber (1900), Tello (1907), Boeke (1916) and Hinsey (1927) in various muscles, especially intercostals and interossei. In all of these studies re-innervation was allowed to occur before atrophy had become extreme. Some new fibres were seen making end-plates of a motor type. Others passed around in the periaxial space, some of them, especially in Boeke's series, becoming very much like the normal annular sensory fibres. It would be interesting to discover whether these endings are attached to motor and sensory fibres respectively, and if so how they come to reach their characteristic positions and structure. It is possible that we have here a case in which the nature of the termination is determined by the ingrowing nerve fibre and not by the terminal tissue. But unfortunately there has been no thorough investigation of this problem, nor of the still more important question of whether these regenerated endings become functional.

Re-innervation of the skin. The return of subjective sensory phenomena during recovery from a nerve injury has been frequently studied, but there has been relatively little correlation of these findings with histological studies. Perhaps it is for this reason that there remains considerable controversy about the whole process. Special difficulties are introduced in the study of the skin by the fact that the fibres reach their endings only after passage through a complicated plexus. The plexus is continuous over the whole surface and there is thus opportunity for overlapping of the areas innervated by two nerves. Recovery of sensation in an area which is anesthetic immediately after injury to a nerve may therefore take place in one or all of three ways. 1. By re-adjustment of the function of the fibres in the zone of overlap between the distribution of two nerves. 2. By local extension of the areas innervated by neighbouring nerves,

due to growth of new fibres through the plexuses. 3. By return of fibres to the denervated area by regeneration of the injured nerve. We may now discuss these processes separately, although in an actual case it is not always easy to distinguish them.

1. At the margin of a denervated area there can usually be recognised an "intermediate zone" containing fibres from a neighbouring nerve. These fibres are not always able to function in isolation immediately after the nerve section, so that the area of sensory loss is greater immediately after injury than, say, two weeks later. It is not clear whether the adjustments which are responsible for this rapid recovery take place peripherally or centrally (see Weddell et al., 1941).

2. The whole area reached by the fibres of a nerve is known as its *maximal zone*. Following recovery in the *intermediate zone* as described above, there remains anesthesia only in the *autonomous zone* of the nerve. Recovery in this latter zone may however take place without the growth into it of fibres which have proceeded from the point of injury down the nerve trunk. Some measure of recovery can take place by the growth of fibres into the area from the unsevered fibres belonging to adjacent nerves (Weddell and Glees, 1941; Weddell et al., 1941). It is not obvious why these fibres should grow out, since their axons have not been interrupted, but it is suggested that degeneration and new growth are continuously taking place in the plexuses and that this innervation by local extension is merely the manifestation of these powers of outgrowth. It seems not unlikely that the denervated portion of the plexus actually provides some stimulus to outgrowth from neighbouring regions. Presumably fibres from these regions would not normally be able to reach out into the autonomous zone of another nerve. Similarly Fort (1940) found that extra nerves implanted into the sartorius muscle of toads can only innervate the muscle fibres if the normal nerve is cut. Although recovery by local extension is probably not able to affect large areas of skin it can certainly produce full return of sensation to a small area. Thus Gutmann and Guttman (1942) have seen complete recovery of algæsia in the area of the heel innervated by the sural nerve of young rabbits, after removal of a stretch of that nerve in such a way as to prevent re-union of its stumps. In older animals recovery was only partial.

3. In spite of the complicating factors introduced by the presence of intermediate zones and recovery by local extension, the sensory recovery through fibres growing down the nerve trunks proceeds in general from above downwards along an area, though the margin of advancing sensibility is seldom straight, and its progress may be irregular. In small areas recovery may be from the sides of the area inwards, or even from below upwards. But in larger areas, and especially toward the ends of the limbs, where there is less complication from neighbouring nerves, the forward progress can regularly be recorded (see Bunnell and Boyes, 1939). By careful study of recovery of response to pin prick on the foot of a number of rabbits it was found that the average rate of advance of the margin of algæsia was 2.05 ± 0.14 mm/day after crushing the peroneal nerve, somewhat less after its suture (Gutmann et al., 1942). Rates not very different

can be calculated from the data of Trotter and Davies (1909) for Man. Such estimates must of course be distinguished from those for the rate of advance of axon tips or of medullation in the nerve trunks. In the present case what is estimated is the rate of advance of the process of functional completion in the cutaneous plexuses, and this rate may appear to be low because the actual distances to be travelled are greater than those measured on the skin. The rate of maturation may however well be actually lower in the finer branches of the plexus than it is in the main trunks.

Modern work on the subjective phenomena of sensory recovery may be said to begin with the postulation by Head, Rivers and Sherren (1905) of a period of protopathic sensibility, early in recovery, before the finer, epicritic, powers return. No agreed histological basis for this distinction has ever been reached, and the possibility of subjective separation of periods in this way has been denied by Trotter and Davies (1909) and several others. There is even considerable disagreement as to the order in which the various modalities of skin sensation return. The most usual order is for pain to return first, then touch and cold, with warmth somewhat later, but the order may vary somewhat. Now that the sensory functions of the various types of ending in the skin are becoming clear (see Woollard et al., 1939) we may hope for further information about the correlation between the peculiarities of sensation during the early period of recovery and the irregular shapes of the newly-formed endings.

Degeneration and regeneration of the sensory corpuscles in the skin follows the same general pattern as that of the motor end-plates. There are some changes during the period of denervation, but usually no complete atrophy of the end-organ, though this may occur in some cases, for instance with taste buds (see Boeke, 1935) and perhaps would also be found to occur in areas left denervated for very long periods. Then during re-innervation there is at first an excess of new fibres and these make atypical endings. In addition new sensory corpuscles may be formed. Sasybin (1930) has made a careful study of these phenomena after removal of pieces of skin and after skin transplantation.

Much of the best work on this subject during the past two decades has been on the large sensory corpuscles of Grandry and Herbst in the bill of the duck. Tamura (1922) found shrinkage of the cells of these organs 5-8 days after denervation. The first new fibres to enter the Grandry corpuscles make an irregular network which becomes normal after 40 days. Boeke (1923) also showed that these corpuscles do not degenerate, but there is perhaps some increase in the number of their nuclei. During re-innervation the fibres at first tend to grow round, rather than within, the corpuscle, but in 2-3 months they become normal. He believes that many new corpuscles, especially Grandry's, are produced. Dijkstra (1933) maintained the denervation for four months, but found very little atrophy of the corpuscles. During re-innervation he did not find the abnormal forms seen by Tamura, but he agrees that new organs are formed, and showed that they form in the scar after the removal of a whole area of skin. He also transplanted skin from the bill of the duck to the leg and vice versa. The skin of the leg normally contains few corpuscles of Grandry, and during

re-innervation of the piece transplanted to the hill none were formed. In the piece from the bill, however, there was both re-innervation of the old corpuscles and the production of new ones. This important experiment confirms the conclusion that re-innervation is mainly a non-specific process of outgrowth "at random", and shows that, at least in this case, the nature of any new organs which are formed under the stimulus of re-innervation is controlled by the tissues and not by the ingrowing nerve fibres.

In the human skin Bocke and Heringa (1924) studied a piece taken nine months after nerve suture from an area showing "typical protopathic sensibility." Some only of the Meissner corpuscles were innervated, but all those of Ruffini and Golgi-Mazzoni. Innervation of the hair follicles was plexiform and very abnormal.

A most careful study of recovery in the skin of the finger tips of monkeys has been made by Jalowy (1935). During degeneration he found changes in the end-organs, but only some of them disappeared altogether. Neither the Merkel nor the Vater-Paccinian corpuscles showed any gross degeneration, though they sometimes stained abnormally. After 25 days many of the cells of the Merkel corpuscles become multipolar; though he does not mention any nuclear proliferation in them yet he calls them quasi Bünchner's bands. By the 40th day many Merkel corpuscles have been absorbed so that only half of them are fit for re-innervation, which proceeds gradually, with many irregular forms in the early stages. Some epithelial cells are converted into Merkel corpuscles, but many of the nerve fibres at this time make no special sensory endings, and there is an excess of "intra-epithelial fibres." In a piece of skin examined 270 days after operation this excess of free endings had been reduced, but there were still many abnormal whorls in the skin and only some of the Merkel and Meissner corpuscles were normal.

Studying re-innervation of the skin of the snout of the guinea pig Jalowy (1934) found similar phenomena; an early excess of intra-epithelial fibres and of fibres around the hairs. The return of Merkel's corpuscles to normal was gradual, in particular the loops which link the corpuscles together appeared very late.

There is still no published case in which the condition of the end-organs in the skin has been thoroughly correlated with functional recovery. It is therefore not yet possible to say how far the excess of innervation and abnormal shapes of the endings are responsible for the aberrations of sensation which are observed. There is every reason to think that the correlations will be found to be close, though the imperfect maturation of the nerve trunks may be equally important in producing these aberrations. The atypical whorls in the skin and the absence of loops between the Merkel corpuscles would be expected to lead to abnormalities in the sense of light touch. The excess of intra-epithelial endings may well be correlated with the curious forms of pain which are noticed. In fact the whole process of normalisation of structure and function in the skin during recovery is well worth further study. The conditions found during regeneration may help to explain such curious phenomena as that reported by

Hogg (1941), who found that during development only free endings may be present at a time when the fetus is already able to respond to light touch.

Since each unit area of skin is normally approached by fibres from more than one direction (Weddell, 1941a, b) it is suggested that the gradual return of sensation is a result of the slowly increasing number of new endings which are completed as fibres grow into an area from various directions. Weddell suggests

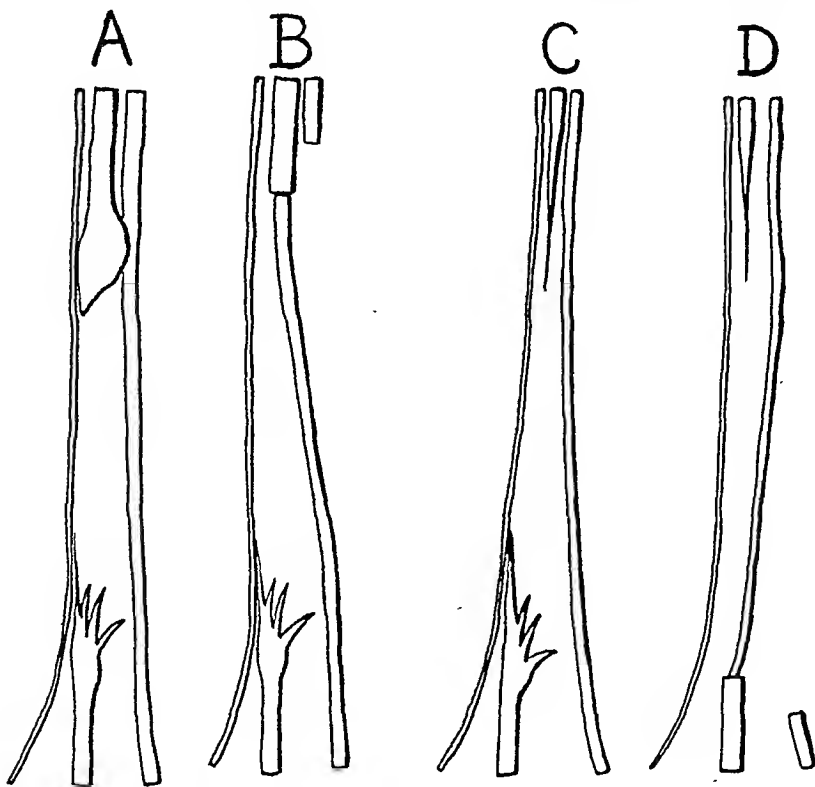


Fig. 8. Experiments to show the effect of delay in making sutures. *A* and *B*, to test the power of outgrowth from the central stump. *A*, the tibial nerve has been cut, several centimeters resected from it and the animal left. Neuroma and glioma have formed. *B*, the neuroma is removed, peroneal nerve cut and its peripheral end sutured to the base of the neuroma.

C and *D*, to test the receptive power of the peripheral stump. *C*, several centimeters have been resected from the tibial nerve and formaldehyde injected to prevent outgrowth from the central stump. *D*, the glioma is removed, the peroneal nerve cut, and its central stump sutured to the base of the glioma.

that the "explosive" sensation observed during the early part of regeneration is a result of the fact that "each sensory spot is innervated by a single fibre instead of multiple fibres". He also suggests that similar disturbances of pattern are responsible for the irradiation of sensation which is felt in intermediate zones.

It is clear that after the first arrival of fibres in the skin there is a considerable period of normalisation before anything like a full recovery is made. We do not yet know enough to say whether this period is occupied by maturation in the

end-organs of the skin, in the plexus, or in the nerve trunks. Perhaps all of these are involved. Further knowledge on these points might point the way to procedures for improving the imperfect recoveries so often recorded in Man.

The effect of delayed suture upon regeneration. Regeneration can take place when stumps are sutured after being left apart for many years. It is generally considered that sutures which have been long delayed produce unsatisfactory recoveries (Lewis, 1920; Foerster, 1929) but it is not clear which of the above variables is responsible. Kilvington (1912) investigated the recovery after delayed suture in four dogs and concluded that the reduced effectiveness of regeneration is due to a decline in the power of the central stump to send out fibres, rather than of the peripheral stump to receive them. We have investigated these factors separately in the rabbit (Holmes and Young, 1942). In order to examine the power of outgrowth from the central stump a stretch was removed from the tibial nerve in the thigh, and the central stump left to form a bulb. After various times a second operation was performed, the bulb removed and the stump sutured into a fresh peripheral stump, provided by cutting the hitherto untouched peroneal nerve (fig. 8, *A* and *B*). The animals were then left for 25 days, at the end of which time the distance reached by new fibres was determined by pinching the peripheral stump (p. 345). The second operation was done at times varying from 2 to 365 days after the first, and in all cases the power of outgrowth was found to be as great as in cases where a primary severance and suture was made, this being done on the opposite sides of the animals as a control. There is therefore, contrary to the belief of Kilvington, a remarkable conservation of the regenerative power of the neurons, even when they are not functioning. Cutting twice at short intervals (2 days) was also found not to reduce the power of outgrowth.

The power of the peripheral stump to receive new fibres can be tested by the converse experiment (fig. 8, *C* and *D*). A large stretch was resected from the tibial nerve in the thigh, and the central stump injected, usually with formaldehyde, to reduce outgrowth (p. 330). The animals were left for varying periods up to 15 months, and the peripheral stump was then exposed, a portion removed from its end, and the hitherto intact peroneal nerve severed and joined to the tibial by means of concentrated plasma. Study of the piece removed showed that the procedure had been effective in preventing the re-innervation of the peripheral stump, except in one or two cases where a few fibres were present. A similar operation on the opposite side of the same animal provided a control primary suture, and the animals were then left for varying periods to enable determination of the distance to which fibres would grow and the time at which they would medullate.

It was found that when secondary suture was made within about six months of the original injury the distance reached by new fibres, as determined by the response to pinching the nerve (p. 345), was as great as with primary sutures. After longer periods, however, the results became irregular, a number of the cases showing reduced distance of outgrowth. Even as long as 17 months after injury, however, it was found that some cases showed regeneration of the full

extent expected. This seems to show that the reduction in the volume of the Schwann tubes, and their complete filling with the protoplasm of the Schwann cells does not present an extra barrier to the outgrowth of fibres. This point has special interest because it suggests that there is not likely to be a decline in the rate of growth of fibres in the distal portions of long nerves, such as those of man, in which the Schwann bands must reach a state similar to that in these experiments before the fibres can reach them after a suture.

The explanation for the variability of the distance of outgrowth recorded after secondary suture was found by examination of the region of the suture itself. In many cases the union with the long-degenerated stump was made less well than that on the control side. This appears to be the result of a failure of outgrowth by the Schwann cells. In some cases it could be seen that the cut surface of the peripheral stump, which normally yields a broad stream of Schwann cells, was closed off abruptly by fibrous tissue, though usually some outgrowth of Schwann cells had taken place. This condition would certainly not be the most favourable for the making of a successful union. In fact if there is a decline in the power of outgrowth from the peripheral stump this by itself constitutes a strong contra-indication to a long delay before performance of suture. In order to investigate this question further, Abercrombie and Johnson (1942) have studied the power of outgrowth *in vitro* of Schwann cells from stumps left uninnervated for various periods. Ingebrigtsen (1916) showed that Schwann cells will not move out from an explant of a piece of normal nerve, but only from one which has been allowed to degenerate for 4 days or more. Abercrombie and Johnson find that this power of outgrowth continues to increase for some time, so that the greatest outgrowth is obtained from explanted pieces of stumps previously degenerated for 19-25 days. After this period the power of outgrowth declines rapidly up to about 60 days, and thereafter more slowly. Even after a year however the outgrowth is much more vigorous than that which occurs from a piece explanted from normal nerve. These experiments thus agree with the histological observation of secondary sutures in showing that when stumps are joined after long delay the union has to be made without the maximum assistance of the cells of the peripheral stump. Nevertheless these cells retain their powers, though to a diminished extent, for a very long time.

The experiments in which delayed suture of the peripheral stump was made provided evidence that medullation is less rapid after secondary than primary suture. This is perhaps also a result of the reduced activity of the Schwann cells in applying themselves to the new fibres. In these stumps which have remained uninnervated for so long, the size of the Schwann tubes becomes very much reduced, and correspondingly more of the cross section of the nerves is taken up by the endoneurium itself. This shrinkage, by approximately one half, reduces not only the diameter of each tube but also that of the whole nerve. This is itself an adverse factor in delayed suture, since it makes it difficult to obtain good apposition of the stumps. We have seen (p. 345) that after primary suture, when the tubes are large, great numbers of new fibres may enter into each old tube. After secondary suture this number is certainly less, though several

fibres may often be seen within even a small tube (fig. 4). It is probable that this reduced "penetrability" of the long-degenerated stump is another factor operating to make secondary suture unsatisfactory.

In the early stages the new fibres are much larger in each tube after delayed than immediate suture, presumably because they meet more resistance to their advance. But this does not lead to more efficient medullation. In stumps examined 100 days after suture there were found to be much larger and better medullated fibres after immediate than delayed suture. In fact the shrinkage of the Sebwann tubes during the long degeneration makes it difficult for the new ingrowing fibres to increase rapidly in diameter, just as it did when a somatic nerve was sutured to a postganglionic one (p. 348). The atrophy of the nerve produced by long degeneration cannot therefore be reversed, at least for a long time, but imposes a limitation on the regeneration which retards it, perhaps indefinitely. The whole diameter of the nerve is in fact still very much smaller at 100 days after secondary than after primary suture.

Finally we may mention here the worst of all effects of delayed suture, namely, the excessive atrophy of the end-organs, and especially muscles, which it produces. This, with all its secondary consequences, is itself alone a strong contra-indication to delay. The muscle fibres shrink rapidly in volume and may disappear altogether, there being proliferation of interstitial fibrous tissue and perhaps actual transformation of muscle into fibrous tissue (Tower, 1939). Moreover if the motor end plates disappear new ones must be made. These changes are accompanied by contracture and fixation of the muscle which makes recovery both of the muscle itself and of associated structures progressively more difficult. In an attempt to measure the effect of this atrophy on subsequent recovery Gutmann (1942) has tested the speed and quality of recovery of atrophied muscles by severing the peroneal nerve of the rabbit in such a way as to leave the peripheral stump uninervated for periods up to 8 months. A second operation was then performed, the tibial nerve being cut and sutured into the long-degenerated peroneal stump, a similar procedure on the opposite side serving as a control. Only partial spreading of the toes ever appears after such cross union but it was found that the onset of recovery was somewhat delayed, and its extent always less, after long degeneration than on the control side. It is not of course possible from these experiments to say to what extent these effects of delay in suturing are due to atrophy of the muscle or whether the factors discussed above, which may be called atrophy of the nerve, play any part.

No method is known by which muscle atrophy can be prevented, but it is increased by stretching and can be reduced by exercise of the muscles with electrical stimulation and perhaps massage. Gutmann and Guttmann (1942) have recently shown that denervated muscles which have been given galvanic treatment maintain their volume much better than untreated ones, and develop less fibrosis. The actual time of the beginning of recovery is not shortened, but the treated muscle weighs much more at the time of recovery and its contractile power develops more quickly. Eccles (1941) has found beneficial effect in disuse atrophy from very short faradic stimulation of the nerve, but the shortest time of

treatment which is effective in atrophy after denervation is not known. The reasons for the atrophy of muscle during denervation are still obscure (see Tower, 1939). When they are revealed it will perhaps be possible to provide effective means of preventing this most serious effect of delay in re-innervation.

Delay in suturing is therefore certain to lead to reduced efficiency of regeneration because 1, the atrophy of the nerve makes apposition difficult; 2, the Schwann cell outgrowth is reduced and the union therefore less well made; 3, the tubes in the peripheral stump being smaller can receive only fewer fibres, giving smaller chances of appropriate connections; 4, medullation is seriously delayed and full maturation of the fibres probably permanently prevented; 5, there is

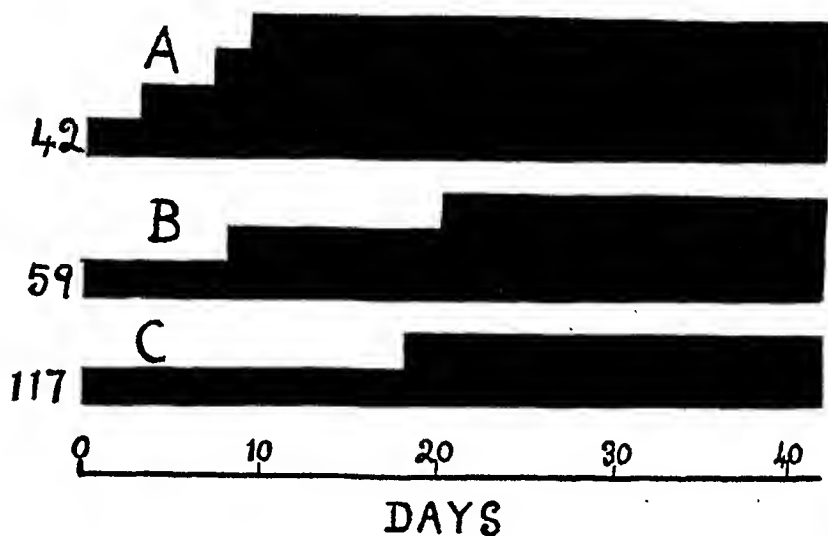


Fig. 9. Diagrams to show the recovery of the function of spreading of the toes of the rabbit after various procedures. Four degrees of movement were distinguished and each diagram shows the improvement recorded during the 6 weeks following the first appearance of recovery. A, after a single local crush; B, after severance and suture of the nerve; C, after removal of 2 cm. from the nerve (unaided union). All the lesions were made at the same distance from the muscle, and the time of the onset of recovery is shown at the left of each figure. (Figure kindly supplied by Dr. E. Gutmann.)

progressive atrophy and ultimately disappearance of muscle fibres, end-plates, and end organs in the skin. It is impossible to say exactly at what time these effects of delay begin to become serious. All the factors except 2 increase progressively from the beginning, and it seems not too much to say, therefore, that any delay greater than, say, one month before the performance of suture must retard recovery and prejudice the final functional result. There are of course many conditions which enforce some waiting in human cases, but the above considerations suggest that the shorter the delay the better will be recovery. Clinical results do not contradict this view.

The factors affecting recovery. A comparison of the actual functional results achieved after various types of nerve injury and suture makes a useful basis for

a final discussion of the factors controlling recovery. Gutmann (1942) and Gutmann and Sanders (1942) have made such comparison possible by producing lesions of various sorts in the peroneal nerve of the rabbit, keeping all other factors, such as distance from the muscle, constant. They have compared the time and extent of recovery of the function of spreading of the toes, reflexly produced by a sudden lowering of the animal. Since this movement involves only a single group of muscles it is possible to make much more reliable comparisons than with study of recovery of some complex movement, such as the gait of the whole limb, which involves the use of normal as well as recovering muscles.

A scale of four degrees of recovery was adopted, 1 indicating the appearance of a very slight movement and 4 full normal function. After interruption of the nerve by thorough crushing with forceps at a single point 80 mm. from the muscle, function began to return between the 40th and 50th day. Thereafter improvement in the extent of the movement was rapid, so that stage 4 was reached a week after the beginning of recovery (fig. 9). Recovery even continued until the movement was *greater* than that normally observed (cf. Machida, 1929). This "overspreading" of the toes usually lasted for a few weeks and then gradually subsided. It would be interesting to discover whether this overfunction is due to the excess of fibres produced. Full normality of recovery was not seen after any other procedure except this simple crushing. After severance and suture at 80 mm. from the muscle function began to return between 60th and 70th day, and slowly improved up to stage 3. No animal ever showed full recovery (stage 4) after suture and many did not proceed further than stage 2. The insertion of autografts 2 cm. long produced results very similar to suture, and homografts were only a little worse. When a gap of 2 cm. was left between the severed stumps (p. 325) function only re-appeared at all in one of six cases, and then on the 117th day. Even after 180 days it reached only the degree represented by stage 2.

When the nerve was crushed completely over a length of 4 cm. the time of onset of recovery was later, and its progress slower than after a crush at a single point, but the final recovery achieved was better than after severance and suture. This is of special interest because crushes of this sort resemble the nerve lesions found in some gunshot wounds.

Gutmann also studied the effect of the level of lesion on the recovery of motor function. After simple crush lesions at the knee the full extent of toe movement returned, on the average, 8 days after the beginning of recovery, whereas after a similar lesion high in the thigh the recovery, besides of course beginning much later, proceeded more slowly and was complete only after 14 days. This difference may be due to the greater degree of muscle atrophy which follows the more distant lesion, perhaps also to the more scattered arrival of regenerated fibres at the muscle and the necessity for them to become more fully medullated in order to function effectively over the longer stretch of new nerve (see p. 352).

Similar results were obtained by Gutmann and Guttmann (1942) in a study of the effect of various factors on the time and degree of sensory recovery, though

it is less easy to make exact quantitative comparison of the degrees of sensory function. The shrinkage of the autonomous zone of analgesia of the peroneal nerve of the rabbit proceeds more slowly after severance and suture than after a local crush (p. 351). The level of lesion also has a considerable influence. The nerve was crushed in different animals at various levels between the ankle and the hip and the time of recovery was, at least in some cases, unduly delayed in the higher lesions. Moreover the advance of recovery over the dorsum of the foot was sometimes quicker after the closer lesions.

From these experiments it is clear that the most important factor determining the time and degree of recovery is the condition at the site of the lesion. The processes of regeneration proceed at their maximum rate, and reach their fullest extent, only when a nerve has been crushed over a very short length. A crush over a long length is followed by a less complete recovery, and if the nerve be cut and sutured then even under the most favourable circumstances the regeneration will be much slower and less complete than after a crush lesion. Any gap left between the stumps produces yet further delay and imperfection of recovery.

Other factors seem to be of smaller importance. Delay in the performance of suture leads to unsatisfactory unions and prejudices the chances of recovery in various ways (p. 366). The distance of the lesion from the end organ also has an effect on the progress of recovery as well as the time of its onset.

For our present purpose the significant features of these experiments are first, that after a crush recovery rapidly reaches a full value, and secondly, that after the other injuries, though it may show a slow improvement for a while, it fails to reach normality. These facts all agree with the thesis that the chief factor which determines the extent of the recovery is the number of initial sprouts from the injured region which regain appropriate peripheral pathways. It would be most important to be able to decide for certain what are the factors which produce the improvement after the beginning of re-innervation, so that we might attempt to assist them and increase the degree of recovery. Weiss (1941a) has produced evidence to show that in the tadpole thick bundles of nerve fibres are formed by successive addition of fibres to successful fasciculi. He found that when a piece of central nervous system and a limb bud were "deplanted" near together into a tadpole's tail, direct connections were formed between them. He suggests that this condition is a result of the fact that of fibres growing out in all directions from the deplanted nerve cells "only those nerves which have entered the limb, have subsequently become filled up into a sizable bundle . . . obviously those pioneering fibres which had accidentally struck the limb had thereby acquired some contact property which made their surface sticky, or otherwise a pathway of preferential application, for other fibres growing out subsequently" (p. 181).

The bundles of fibres which are produced in mammals at the extremity of an isolated central stump, or between the stumps after a bad suture, give every reason to suppose that some similar process of addition is taking place. It seems almost certain that a process of fasciculation by addition takes place also during the outgrowth of Schwann cells from the peripheral stump. But in

both these cases, since no functional connection has yet been formed, it would seem that the outgrowing fibres, whether nervous or Schwannian, tend simply to stick together and to make pathways along which others are added. If this process could continue indefinitely, and especially if new fibres were later added alongside those which made successful functional connections, we should have a mechanism by which the degree of function would be gradually improved.

In the case of the recoveries after local crushing the improvement in function is too rapid to be due to subsequent addition of further fibres to those which have established successful connections. The improvement in that case is probably due rather to the increase in number, diameter and medullation of the fibres, leading them to carry volleys of higher rates and more effective synchrony. These factors are no doubt responsible for part at least of the slower improvement which is seen after suture or grafting. The fact that in these latter cases no full recovery was reached shows that the process of subsequent addition to successful fibres is not rapidly effective, but it may well be that it is at work none the less. It must be remembered that many fibres enter each original tube in the peripheral stump (p. 343) and that we do not know what factors determine which of these shall survive, and increase in diameter. Certainly more than one fibre can become enclosed in the Schwann protoplasm and acquire a myelin sheath, and it is not impossible that once one fibre of a set within a tube has made successful connection others are assisted with medullation. But it is equally possible that the reverse occurs and that the successful fibre eliminates the others. At every step we are faced with the importance of discovering more about the trophic factors which promote the maintenance and increase of some fibres but not others.

It is very probable that the subsequent addition to successful fibres, such as would produce full recovery, depends, as Weiss suggests, on the nature of the medium. Such fasciculation no doubt takes place readily only in a rather fluid medium, which may perhaps be provided during the early stages, but not later, when the tissue between the stumps has become organised.

In Amphibia it appears that even a relatively exiguous nerve supply is able to expand, as it were, by branching, so as to control a large peripheral field. Thus the innervation of a given region of a regenerating limb is equally rich whether it is allowed normal, supernormal or reduced nerve supply (Weiss, 1934; Litwiler, 1938, 1938a). The data available suggest that in mammals the capacity for such adjustment is smaller. When recovery is poor or absent after long periods, as in the above experiments or in an old unrecovered nerve injury in man, one finds few and poorly medullated fibres in the peripheral stump and end-organs. They have not multiplied to supply the whole field. It cannot be excluded that they would do so if given sufficient time, but I have seen such a condition in the peripheral stump of a radial nerve injured 7 years before, when the subject was only two years old.

In fact there is little evidence that in mammals the "demand" of the periphery can increase the supply of fibres. The number in any region of nerve, muscle or skin appears to be determined by the number which have arrived there during

the initial outgrowth along the Schwann cells, and perhaps other surfaces, which are provided. On the other hand there must be potent influences which *reduce* the numbers of superfluous fibres. We have seen these influences at work at many points; in the removal of the majority of that enormous number of fine end-bulbs which is produced in the scar during the early period of outgrowth, in the removal of the spirals of Perroncito, and in the reduction of the number of the fine fibres which at first enter the peripheral stump. Absence of connection, first with any suitable surface in the case of the initial bulbs, and then with a chain of Schwann cells, may be the chief factors which lead to this atrophy. Absence of function may be another factor. However it is certain that nerve fibres can persist without useful function for a very long time. It is impossible to say that either motor or sensory fibres in an amputation neuroma carry no impulses, though if they do carry them it is but uselessly. Yet they may remain for many years. In fact this problem, like most of the problems of trophic dependence and effects of use and disuse, is shrouded in mystery.

Nor is there much evidence that neurons which form atypical connections can subsequently re-adjust their function. Sperry (1940, 1941, 1942) has shown that when the function of a single nerve is examined in the rat it is found that very little adjustment can take place. The fibres of the peroneal nerve continue to carry impulses in the reflex situations in which they would normally be in action, whatever effect the impulses are made to have by transplanting the nerve or the muscle. In Man some adjustment may be possible but the possibility of re-education is very limited. Thus Ford and Woodhall (1938) state that in recovered facial palsies "whenever the patient moved any part of (that) side of the face every muscle supplied by the 7th nerve contracted".

In Mammals, then, there is little indication that recovery is helped by any special agencies such as specific direction of outgrowth, attraction to end-organs, successive addition of new fibres to successful ones, atrophy of wrongly connected fibres or profound central re-adjustments.

Successful nervous regeneration must therefore depend mainly on the chances provided for adequate numbers of the outgrowing fibres to establish connections resembling their original ones. For this purpose it is provided that very many nerve fibres shall sprout out from the central stump and be met by many strands of Schwann cells, reaching out across the scar and thus ready to lead them to the peripheral stump. The axon tips then progress down the nerve, many within each original tube, and some of them will connect with appropriate end-organs. Function will not return with the first arrival of fibres at the end-organ, but only when the full process of regeneration of nerve has been completed by increase in the diameter of the fibres and their medullation. The rate and extent of the increase of fibre size probably depends on the outflow from the central fibre. Hence the diameter of the latter, and perhaps the number of branches which it has to feed, determine the course of regeneration. But the size of the Schwann tube of the peripheral stump also affects the maturation. Small tubes allow at best only a slow increase of diameter, so that the longer a nerve has atrophied the less effectively will it regenerate.

The process of maturation therefore sweeps down the nerve long after the advance of the axon tips, and at a slower rate than the latter. Function only recovers when and to the extent that a sufficient number of the fibres with appropriate connections have become thus functionally completed.

Acknowledgments. I am very grateful to my colleagues in Oxford who have not only made unpublished work available for this review but by discussion and reading of the manuscript have largely contributed to its preparation. Drs. P. Glees, E. Gutmann, L. Guttmann, Messrs. W. Holmes, P. B. Medawar and F. K. Sanders and Dr. G. Weddell have all helped in such ways. I am especially grateful to Prof. H. J. Seddon for the ready access he has given us to clinical material, and for much advice.

The research on peripheral nerve regeneration in the Department of Zoology and Comparative Anatomy at Oxford has been assisted by a grant from the Rockefeller Foundation.

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EXTRAMEDULLARY BLOOD PRODUCTION

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In the case of mammals throughout post-natal life blood cells are produced under normal conditions exclusively in the bone marrow. This statement applies only to two of the three predominating and essential hemocytes, the erythrocytes and the granulocytes. The third type of cell which occurs in great numbers in the circulating blood, namely, the lymphocyte, is produced chiefly in the lymph nodes and the spleen, to a relatively small extent in the bone marrow. It is essentially extramedullary in origin. The lymphocytes formed in lymph nodes enter the blood stream via the lymphatic radicles of the thoracic duct and the right lymphatic duct; those produced in the spleen and the bone marrow pass directly into sinusoidal venous channels. Minor contributions of lymphocytes are made also from the thymus and lymphoid areas in the wall of the gastro-intestinal canal. This mixture of lymphocytes with erythrocytes and granulocytes in the blood, and especially the presence of lymphocytes in the bone marrow, at once raises the questions of the function of lymphocytes and of a possible genetic relationship of lymphocytes to erythrocytes and granulocytes.

Extramedullary blood formation occurs under various pathologic conditions in adult mammals. Recorded cases refer chiefly to man. Brannan (1927) has published an extensive review of the literature on extramedullary hemopoiesis in anemias. Diamond, Blackfan and Baty (1932) and Strong and Marks (1939) reviewed the recent literature on ectopic blood production in erythroblastosis fetalis. The tissues most frequently involved are lymph nodes, spleen and liver. But extramedullary blood formation occurs normally in the fetus in liver, spleen and lymph nodes. It is a significant fact that normal embryonic and fetal extramedullary blood formation involves the same organs in which the phenomenon occurs pathologically in the infant and adult.

When search is made for common anatomical features among red bone marrow, spleen, liver and lymph nodes one finds only a sinusoidal circulation, lymphatic in the case of lymph nodes. But certain other tissues (e.g., erectile tissues, suprarenal cortex, fetal and adult kidney) have in part a sinusoidal venous circulation. In contrast with lymph nodes, where lymphatic sinuses are relatively voluminous, spleen, liver and bone marrow lack definite parenchymal lymphatics. A feature common to spleen, lymph nodes and marrow is the occurrence of lymphocytes, predominant in lymph nodes and spleen, normally relatively sparse in mammalian red bone marrow. Anatomic conditions favorable for red cell production include sinusoidal venous circulation, lack of lymphatics and presence of lymphocytes. Only in the older fetus and, under pathologic conditions in the infant and adult, are erythrocytes produced in lymph nodes. In the adult the evidence indicates that in erythrocytopoietic lymph nodes the afferent or efferent lymphatics or both have become disconnected. Certain sinuses may become connected with the blood vascular system, as in

hemal nodes, or fluid exchange may occur between the blood and the stagnant lymph of the sinuses, effecting a close physiologic identity. Pathologic lymph nodes with erythroid metaplasia thus correspond closely to conditions in spleen and bone marrow. Even under normal conditions lymph and blood plasma are closely similar in chemical composition.

Comparative data. In lower vertebrates blood formation is exclusively extramedullary. Hollow bones, with erythropoietic marrow, appear first in anuran amphibians. In the tailed amphibians (salamanders) red blood cells are produced in the spleen. Even in the tailless amphibians the bone marrow functions erythrocytopoietically only for brief periods following metamorphosis and the annual hibernation; during the remaining time red cells are produced in the spleen. Prior to metamorphosis red cells are produced also in the venous sinusoids of the mesonephros. In the higher sub-mammalian vertebrates (reptiles and birds) erythrocytes are produced in the bone marrow; also in the spleen to a considerable extent in certain reptiles, to a small extent in birds.

It becomes apparent that phylogenetically the spleen is an important red cell producing organ; indeed, in fishes it is the primary blood producing organ, with variable assistance from the mesonephros. It appears that red cell formation in bone marrow is a secondary, essentially fortuitous condition. With the appearance of hollow bones, with a vascular nutritional medulla, conditions in the marrow, with its sinusoidal venous circulation, became favorable for red cell production, and this activity was shifted from spleen to bone marrow (Jordan, 1933, 1937).

In the lowest fishes, e.g., the hagfish, the spleen consists of loose lymphoid tissue, enveloping the veins of the submucosa, along the entire length of the short intestine. In the lymphoid tissue enveloping the terminal thin-walled venous channels certain cells enlarge and migrate into the venous sinusoids, where they elaborate hemoglobin and mature as erythrocytes. The spleen of the hagfish is the locus of origin also of granular leucocytes; these develop in situ from enlarging cells in the extravascular lymphoid parenchyma. This lymphoid tissue in the wall of the intestine of the hagfish may be designated a "disperse spleen." In the lamprey, moderately compact lymphoid tissue occurs in the spiral valve of the intestine. This lymphoid tissue constitutes a "diffuse spleen." As in the spleen of the hagfish, certain of the lymphoid cells enlarge and migrate into adjacent venous channels. These intravascular hemoblasts differentiate into erythrocytes; identical extravascular hemoblasts develop into granulocytes. In the lungfish, lymphoid tissue becomes aggregated in the wall of the stomach, constituting an "aggregate spleen." Here both red and white cells differentiate, red cells in venous sinusoids, granulocytes in intervascular stroma.

In the ganoid fishes and in elasmobranchs, lymphocytes become segregated into a discrete encapsulated spleen attached to the gut by a distinct mesentery. In these fishes red cells are formed chiefly in the spleen, to a small extent in the mesonephros. Granulocytes may be produced also in the submucosa of the gut, and in the stroma of the kidney. In elasmobranchs, granulocytes are formed in

the stromal tissue of the ovary and testis. In certain salamanders, granulocytes are produced in the subcapsular area of the liver. The Proteidae are an exception; in these urodeles granulocyte production is limited to the intertubular stroma of the mesonephros, erythrocytes are produced in the spleen (Jordan, 1932). The primary elements of the circulating blood are hemoglobin bearing cells, the erythrocytes; they are the respiratory elements. Lymphocytes, monocytes and granulocytes are in a sense extraneous elements, invasion products, which have entered the blood system for purposes of more rapid transportation.

Considering extramedullary blood formation—whether as a normal process in adult lower vertebrates, a normal process in larval higher vertebrates, a normal process in the mammalian embryo and fetus, or as a metaplastic phenomenon in adult mammals under certain pathologic conditions—with a view to disclosing common factors operative for red cell maturation, whether in primitive kidney (mesonephros), spleen, yolk sac, liver, lymph nodes or red bone marrow, we find two elements common to all the areas: lymphocytes and a sinusoidal venous circulation, that is to say, lymphocytes in a slowly moving plasma with a relatively high CO_2 tension.

The problem, then, in an effort to explain erythroid metaplasia in spleen, liver and lymph nodes in mammals and man, becomes one of determining the genetic relation between lymphocytes and definitive blood cells, both red and white. Very fortunately there exists a most favorable material for study of the problem in the bone marrow of birds (Jordan, 1935, 1936, 1937). Birds lack lymph nodes. Presumably in compensation for this lack the bone marrow contains numerous nodules of lymphoid tissue. These nodules have an extensive supply of capillaries. The lymphocytes are predominantly of the small and medium-sized varieties. Such grow to the size and acquire the cytologic features of hemocytoblasts. Some of these cells enter the capillaries, both by migration and direct incorporation following local transformation of reticular cells into endothelium, whence they are carried into the extranodular venous sinuses with stagnant blood where they mature into erythrocytes. The hemocytoblasts which migrate into the extranodular intervaseular stroma differentiate into granulocytes. Here, then, best illustrated in common fowl and turkey, is a demonstration that lymphocytes function as common progenitors of both erythrocytes and granulocytes. The specific line of differentiation depends upon a differential environment. The fundamental differential factor is the presence of something within the slowly moving blood of the marrow sinusoids that is absent, at least not present to the same degree, in the intervaseular stroma. This something is obviously, at least in part, a difference in the degree of CO_2 tension. Hemoglobin elaboration may be assumed to be dependent fundamentally upon a relatively high CO_2 tension.

The same explanation may be applied in common to tissues where red cells are formed, whether in lower vertebrates, larvae of higher vertebrates, mammalian embryos and fetuses, normal red bone marrow of adult mammals, and metaplastic erythroid areas of adult mammals under pathologic conditions, i.e., to the disperse spleen of the hagfish, the diffuse spleen of the lamprey, the aggregate

spleen of the lungfish, the segregate spleen of ganoids, elasmobranchs and teleost fishes, the spleen of adult amphibians, the mesonephros of larval amphibians, the bone marrow of reptiles, birds and mammals; the yolk sac, liver, spleen and bone marrow of mammalian fetuses; and the liver, spleen and lymph nodes of man under certain pathologic conditions characterized by erythroid metaplasia. In every instance the red cells are produced from ancestral lymphocyte-like cells in venous sinuses with relatively stagnant blood (Jordan, 1939).

In the case of chicken, pigeon and turkey careful detailed comparative studies of the predominating parenchymal cells of the nodules of the bone marrow, spleen and rectal ceca reveal that these cells are cytologically identical. If the prevailing small lymphoid cells of the spleen and the nodules of the ceca of birds are genuine small lymphocytes, then the small cells of the lymphoid nodules of the bone marrow must also be regarded as genuine lymphocytes. Since these same cells in the spleen of the young bird, and homologous cells in the spleen of reptiles, amphibians and fishes, differentiate in part into erythrocytes, it should not be surprising that as a resident of the favorable venous sinuses of the bone marrow the lymphocyte should here also express its potentiality for erythropoiesis. Moreover, the identical cell of the marrow nodules of local primary heteroplastic origin should logically have the same capacity.

The objective evidence seems positive regarding the normal erythrocytogenic function of the small lymphocytes in the bone marrow of birds. Applying the conclusions built upon the data of comparative hematology, more especially those from hemocytopoietic tissues of amphibians and birds, to the mammalian group, the very strong inference is suggested that the small lymphocytes of mammals are also polyvalent embryonal cells which, filtered out in the bone marrow, function as supplementary blood-cell precursors in addition to local ancestors.

EXPERIMENTAL. The intimate functional relationship of the spleen to the process of blood cell production, even in higher vertebrates where erythropoiesis is normally restricted to the bone marrow, is revealed in splenectomized pigeons (Jordan and Robeson, 1942). Pigeons, in contrast with the chicken and turkey, normally lack definite lymphoid nodules in the marrow. Presumably the supply of small lymphocytes from the spleen, to be filtered from the blood stream in the marrow, is adequate for the needs of normal intramedullary erythrocyte production without the help of intramedullary lymphoid nodules. When the greater part of the spleen is destroyed by cauterization the marrow of the femur and the tibia produces its own lymphoid nodules, presumably in compensation for those removed with the spleen. These intramedullary lymphoid nodules, which aid in the supply of ancestral lymphocytes for erythrocytes and granulocytes, in addition to general hypertrophy of the hemopoietic parenchyma, appear towards the end of the third week following subtotal splenectomy and reach a maximum in number and size during the fifth and sixth week. Meanwhile the splenic remnant may regenerate rapidly. In those splenectomized pigeons in which the spleen has regenerated to approximately normal size by the end of the fourth week following the operation, the newly formed lymphoid nodules of the marrow undergo regression. The regenerating splenic tissue itself shows numerous large and very active lymphoid nodules.

The close functional relationship between spleen and bone marrow in birds is in accord with expectations based upon evidence derived from a study of erythrocytopoiesis in amphibians, where the spleen is the chief locus in the adult of red cell production. Confirmatory evidence of the activity of lymphocytes as red cell progenitors in amphibians accrues for the results of inanition experiments with the common salamander, *Triturus viridescens* (Jordan, 1938). When this urodele is starved for a period of about four months a rapidly fatal anemia develops. Persistent erythrocytes are pale and degenerate, and the number of circulating lymphocytes greatly reduced. Furthermore, the spleen has become small and fibrotic through loss of lymphocytes and reduction of proliferative activity. Death can be prevented by feeding with earthworms. Within a week the blood has become restored to normal, except that the majority of the lymphocytes are of the small variety and a large proportion of the erythrocytes are of relatively small size. The interpretation of the presence of these numerous microcytes would appear to be in terms of the predominance of small lymphocytes. The need for new erythrocytes in the terminal stages of the experimental inanition is so intense that the rapidly proliferating ancestral lymphocytes under starvation conditions are not given time or adequate nutrition to grow to the usual size of large lymphocytes before transformation into erythrocytes.

A fundamental question concerning red cell production relates to the primary factor responsible for the transformation of lymphocytes into hemoglobiniferous erythrocytes. Secondary factors comprise basic morphologic and physicochemical conditions necessary for the successful operation of the fundamental stimulating agent, such as intravascular location with slow blood flow or favorable relation to transudations from the blood plasma. The primary factor inheres in a specific stimulus which controls increase in erythrocytopoietic activity. Important converging evidence concerning the identity of the fundamental erythrocytopoietic stimulus accrues from certain experiments with frogs. When frog larvae are treated with thyroid extract the erythrocytopoietic activity of the spleen is markedly increased (Jordan and Speidel, 1923). The mechanism involved appears fundamentally to be an increase in general metabolic rate, which leads to increased formation of acid metabolites, including principally carbon dioxide, with resulting increased hydrogen ion concentration. Helff (1923) has shown that the carbon dioxide-oxygen exchange increases steadily during the first week or ten days after thyroid treatment. Our histological studies of the spleens of thyroid treated tadpoles showed a marked increase of erythrocytopoietic activity during this time. Increased concentration of carbon dioxide acts as a hormone to stimulate more rapid respiratory rate and more rapid red cell differentiation. Both of these rate changes represent merely two aspects of the general physiological adaptation of the animal to the new high metabolic level. The increased metabolic rate, with the resulting increased respiratory and erythrocytopoietic rate, involves more rapid internal respiration, more efficient transport of oxygen and carbon dioxide between respiratory organ and body tissues, and finally more efficient external respiration. The erythrocyte furnishes the chief material basis in each of these processes.

The tempo of erythrocytopoiesis in the frog is influenced also by the seasonal temperature changes. In winter, during hibernation, the spleen is relatively quiescent. During this period the carbon dioxide output is relatively low. With the onset of spring the average level of body temperature rises, bringing about an increase in metabolic rate. This initiates the same chain of reaction described for thyroid administration: increased carbon dioxide concentration, increased respiratory rate, and increased erythrocytopoiesis. In the transition period from summer to autumn the body temperature falls and the metabolic rate in consequence also falls, respiration slows down and the erythrocytopoietic tempo decreases. Histologic examination of the winter spleen reveals fibrotic nodules, representing the remains of active lymphoid nodules of the spleen (Jordan and Speidel, 1923, 1925).

Similar erythrocytopoietic changes result from experimental hemorrhage or red cell destruction by administration of saponin. Aspiration of blood directly from the heart of the frog causes an erythrocyte and plasma deficiency which results in a stimulation of the rate of red cell production. Both erythrocytes and plasma function in the transportation of carbon dioxide. A deficiency of cells and plasma in consequence leads to a greater concentration of carbon dioxide, with resulting stimulation of the erythrocytopoietic tissues, until a more normal carbon dioxide balance is regained (Jordan and Speidel, 1923).

In these several experiments with frogs the end results of the different extraneous stimuli of thyroid extract, temperature change and blood depletion, have involved the production of a common factor, namely, a variation in the concentration of carbon dioxide in the blood. It is this change in carbon dioxide concentration which is believed to be the more immediate fundamental stimulating cause of change in erythrocytopoietic rate. The experiments of Dallwig, Kolmer and Loevenhart (1915) reveal that carbon dioxide concentrations of from 0 per cent to 1.0 per cent cause some stimulation of the bone marrow. They suggest that the mechanism of this stimulation is to be found in the acid properties of carbon dioxide and its consequent power to decrease oxygen fixation. The decrease in oxygen tension of the respired air is followed by increased carbon dioxide accumulation. The oxygen lack in the respired air would be the more remote factor, the carbon dioxide in the body fluids the more immediate factor in stimulating increased erythrocytopoietic activity in the bone marrow (Jordan and Speidel, 1924).

Extramedullary blood formation in man under pathologic conditions. Illustrative of the phenomenon of extramedullary red cell formation in man under certain pathologic conditions, several typical cases may be cited. In a case of microlymphoidcytic leukemia, the lymph nodes were very active in the production of erythrocytes. The patient was a forty-year-old white woman, with enlarged spleen, a red cell count of about 1,000,000, and a leukocyte count of 70,000, of which 83.3 per cent were small lymphocytes. With one radium treatment of 3200 mgm.-hours the leukocyte count fell to 3,200 in six days, the red cell count to 800,000. Death followed four days later.

Briefly sketched the case may be interpreted as follows: The dysfunction

the marrow, the primary cause of which remains unknown, resulting chiefly in a condition of severe anemia, stimulated a compensatory reversion of the spleen to its embryonic hemocytopoietic condition. Relatively intense proliferative activity or inability of sufficiently rapid differentiation of splenic polyvalent lymphocytes into erythrocytes (due possibly, at least in part, to a lack of favorable conditions for the development of hemoglobin) caused an accumulation of the lymphocyte red-cell ancestors, with a consequent enlargement of the spleen. Radium irradiation then destroyed large numbers of the splenic lymphocytes, with a resultant decrease in the size of the spleen and the production of the histologic condition of large areas of lymphocyte-free stroma. Following this intentional destruction of the lymphocytes of the spleen, compensation was attempted on the part of the only other available potentially myeloid tissue, namely, the lymph nodes. There was no evidence of red cell production in the liver (Jordan, 1934).

In a case of adenocarcinoma of the prostate, where the bone marrow was practically completely displaced by metastatic tumor tissue, and where the majority of the lymph nodes had likewise been displaced by tumor cells, the spleen was especially active in erythrocyte production (Jordan, 1934). Also, in those lymph nodes which had escaped metastatic invasion or in some which were only partly displaced by tumor cells, erythrocytopoiesis was active in the lymph sinuses. Red cell production was active also in the sinusoids of the liver. This case illustrates the reversal of the phylogenetic history of the blood-forming tissues. In phylogeny the fundamental blood-forming organ, the spleen, apportions its functions of lymphocytopoiesis and erythrocytopoiesis at the higher levels, respectively, among lymph nodes and bone marrow. The spleen retains prominently in the mammalian adult only the functions of lymphocyte and monocyte formation. Since the lymph nodes also perform these functions to a high degree, the spleen represents as regards its primary function of blood formation in a sense only a vestigial organ. However, by virtue of its reticular stroma, its lymphocyte parenchyma and its sinusoidal venous circulation, it retains its evolutionary and fetal potentiality for the formation of hemoglobiferous cells. In this case of adenocarcinoma of the prostate both the lymph nodes and the bone marrow were largely eliminated from the hemocytopoietic system by reason of extensive metastases, and the spleen was stimulated to assume as a compensatory measure its original erythrocytopoietic activity, and circulating lymphocytes were filtered out in the liver where they matured into erythrocytes. The spleen was spared from metastatic invasion by cancer cells presumably because of the absence of lymphatics in the splenic parenchyma. The condition roughly parallels the evolutionary level of the Amphibia in which the bone marrow has only slight erythropoietic activity, the spleen being the dominant organ in the production of red cells. In amphibians erythrocytes mature also in the general circulation. Histologic and mechanical conditions in the venous sinuses of the marrow of mammals are very similar to those in the spleen and peripheral circulation of amphibians, these locations with slowly moving blood presenting the essential requisite features for erythrocyte

production. From another point of view, this case represents a natural experiment in which most of the lymph nodes and large portions of the bone marrow had been eliminated from the hemopoietic system. The resulting condition provided the stimulus for the compensatory hyperfunction of the remaining, essentially erythrocytopoietic tissue, namely, the spleen.

These two cases in a sense complement each other. They represent natural experiments in which in one case the bone marrow and the spleen had been largely removed from the hemopoietic system, leaving the lymph nodes for compensatory erythrocytopoietic function; in the other case the marrow and the lymph nodes had been replaced by tumor tissue, leaving the spleen unimpaired. In both cases the small lymphocyte functioned as the ancestral cell of the erythrocyte.

Further evidence that lymphocytes have the capacity to function as erythrocyte ancestors in man accrues from a case of aplastic anemia (Jordan, 1933). The patient was a white woman, fifty-six years of age. After three blood transfusions the red cell count was 3,000,000; the leukocyte count was 4,000, small lymphocytes accounting for 65 per cent. Two additional transfusions were given on November 8 and 14, respectively. On November 24 the red cell count had dropped to 800,000. Death occurred on November 25. Lymph nodes, spleen and bone marrow were uniformly hypoplastic. They were functionally exhausted. They had an essentially identical lymphoid structure; the predominating cell was the small lymphocyte.

An especially interesting example of extramedullary blood formation in man concerns a case of osteosclerosis with leukemia (Jordan and Scott, 1941). Here the hemopoietic marrow was practically non-existent. The marrow spaces were filled with fibrous connective tissue. This patient had a red cell count of 4,125,000 and a white cell count of 25,000 the day before death from right-sided heart failure. Obviously red cells were formed in abundance somewhere. Study of tissues removed at autopsy revealed that red cell production was very active in the liver, spleen and lymph nodes. Sections of lymph nodes had almost the identical appearance of active red marrow with innumerable megakaryocytes.

The features of special interest in this case concern: 1, practically complete absence of hemopoietic tissue in the bone marrow, in conjunction with a substantially normal red cell count; 2, hyperplasia of bone in relation to marrow fibrosis (myelofibrosis, myelosclerosis); 3, extensive extramedullary blood formation; 4, "division of labor" as regards hemopoiesis, in that erythrocytes were formed in lymph nodes and liver, and granulocytes in the spleen; 5, evidence that lymphocytes served as common ancestors for both erythrocytes and granulocytes.

The histologic evidence suggests that there was operative some unknown primary factor favoring widespread fibrosis. This factor expressed itself first in the bones where it produced extensive osseous hyperplasia with a concomitant fibrosis of the medullary stroma (myelofibrin) and a disappearance of hemopoietic parenchyma. Since the red cell count remained close to normal, con-

compensation for the disappearance of hemopoietic tissue of the bone marrow must have been found in some of the extramedullary potentially hemopoietic tissues: spleen, liver and lymph nodes.

Study of microscopic sections of these organs revealed that all three tissues were actually active in blood formation; lymph nodes and liver in red cell production, the spleen in granulocyte production. At death many of the lymph nodes were completely fibrotic; some remained in a condition of active erythroid metaplasia. A period must have come in this progressive fibrosis of the lymph nodes when this tissue could no longer adequately supply large numbers of red cells. At this point it was presumably the spleen that undertook the work of furnishing ancestral cells for hemopoiesis.

The spleen became very actively hyperplastic and enlarged to more than five times normal size. But conditions in the spleen were favorable only for granulocyte maturation. We may suppose that the extensive hyperplasia and concomitant fibrosis compressed the venous sinuses, generally favorable for compensatory extramedullary red cell maturation, to a degree where conditions were no longer suitable for erythrocytopoiesis. The enormously excessive number of lymphocytes produced by the spleen, during the period of its great enlargement, entered the circulation and were filtered out in the hepatic capillaries, where they served as ancestors for the production of erythrocytes. These hepatic capilliform sinusoids, areas of slowly moving blood with a relatively high carbon dioxide tension, were apparently similar in essential erythrocytopoietic factors to those of bone marrow, and could accordingly provide compensatory areas for red cell production.

The results of this study give further support to the interpretation of the lymphocyte as an embryonal cell with multiple developmental potentialities, including most prominently that of serving as a common ancestor (hemocytoblast) for the several varieties of blood cells: Erythrocytes, granulocytes, monocytes, plasma cells and megakaryocytes. In the extramedullary sites of blood formation, lymph nodes, spleen and bone marrow, the small lymphocytes grew to the size of large lymphocytes, meanwhile acquiring the cytologic features of hemocytoblasts. Such hemocytoblasts differentiated into erythrocytes within the sinusoidal channels with relatively static blood high in carbon dioxide content (venous sinusoids of marrow, liver and modified lymph nodes), and into granulocytes in the intervacular parenchymal areas.

The data from comparative hematology, the evolutionary history of blood producing tissues, studies of ectopic hemopoiesis under pathologic conditions in man, studies of the fetal tissues involved in normal blood formation in mammals and man, and the results of an experimental analysis of hemopoiesis in amphibians, are all consistent in support of the conclusion that the spleen, liver and lymph nodes may undergo erythroid metaplasia in compensation for hemopoietic dyscrasias in the bone marrow; and that in each of these tissues under all conditions the progenitors of the blood cells are primarily either lymphocytes or the ancestral reticular connective tissue.

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